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(71) Applicant *(for all designated States except US)*: THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY [US/US]; 3601 Spruce Street, Philadelphia, PA 19104-4268 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ERTL, Hildegund, C., J. [DE/US]; 1929 West Montgomery Avenue, Villanova, PA 19085 (US). THURIN, Magdalena [US/US]; 28 University Mews, Philadelphia, PA 19104 (US).

(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).

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(57) Abstract

Methods for inducing an immune response to a tumor cell are disclosed for use in the regression of existing tumors and in the prevention of cancers, especially in high risk subjects with familial histories. Compositions for use in these methods include particularly, DNA vaccines or recombinant viruses which contain a p53 gene which encodes a biologically inactive form of p53 incapable of tetramerization, or which contain a gene encoding a p53 antigen fused to a signal sequence which retargets the protein to preferred cellular compartments. The methods and compositions are further enhanced by co-administration of one or more chemokine, cytokine or combinations thereof.

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METHODS AND COMPOSITIONS FOR INDUCING A PROTECTIVE IMMUNE RESPONSE TO CANCERS

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Field of the Invention

The present invention relates generally to the field of cancer therapeutics and prophylaxis, and specifically to compositions and methods for inducing in an animal or human an immune response to tumor antigens which causes regression of existing tumors and/or prevents the development of cancers, e.g., in high risk patients.

Background of the Invention

Vaccination is the most effective medical intervention to reduce human morbidity and mortality. While vaccines have been used for decades successfully to reduce the incidence of infectious diseases, efficacious vaccines to cancer have been scarce, partially due to a lack of suitable "tumor specific" antigens. Also, cancer cells often evade immunosurveillance by down-regulating determinants crucial for recognition by T cells. For example, the expression of major histocompatibility complex (MHC) class I determinants is commonly reduced or even lost in cancer cells thus affecting recognition by cytolytic T cells [F. Garido et al, Immunol. Today, 14:491-499 (1993)]. Tumors can lack other molecules needed for antigen processing such as the LMP-2/LMP-7 proteosomes or the TAP-1/2 transporters [B. Seliger et al, Immunol. Today, 18:292-299 (1997)]. They can subvert the immune system by secretion of cytokines such as IL-10, transforming growth factor (TGF)-ß, or prostaglandin E [M. Matsuda et al, J. Exp. Med., 180:2371-2375 (1993) and H. Chung et al, Cancer Res., 53:4391-4398 (1993)].

One of the most frequent abnormalities of human tumors are mutations or marked overexpression of the p53 protein, either of which can be found in more than 50% of the most common types of human cancers. Wild-type (wt) p53 is a sequence-specific DNA binding protein found in humans and other mammals, which has tumor suppressor function [See, e.g., Harris, Science, 262:1980-1981 (1993) and A. Levine et al, Nature, 351:453-456 (1991)]. The naturally-occurring wild-type p53 protein functions to regulate cell proliferation by curtailing cell cycle progression and inducing apoptotic cell death upon severe DNA damage. Mutations of p53, which cluster in well-defined hot spots of the gene, lead to structural changes and functional inactivation of the p53 protein. Most mutations prolong the half-life of p53 resulting in functional over-expression. In some types of tumors wild-type p53 is overexpressed to compensate for other abnormalities of cell cycle control or it is abnormally processed, e.g., due to binding to viral oncoproteins such as the E6 protein of human papilloma virus (HPV)-16 [B. Werness et al, Science, 248:76-79 (1990)].

The nucleotide and amino acid sequences of human p53 are known [Zakut-Houri et al, EMBO J., 4:1251-1255 (1985); GenBank Code Hsp53]. The amino acid sequence of p53 is conserved across evolution [Soussi et al, Oncogene, 5: 945-952 (1990)], suggesting that its function is also conserved. The murine cDNA coding sequence of p53 is also reported in GenBank [see, also, Matlashewski et al, EMBO J., 13:3257-3262 (1984) and International Patent Application No. WO94/02167].

A self protein present in excessive amounts in malignant cells, such as p53, can trigger a T cell-mediated immune response by exposing so-called cryptic epitopes. Cryptic epitopes are epitopes that at physiological expression levels have too low an affinity for MHC determinants to reach the threshold needed for induction of T cell tolerance or activation [E. Sercarz et al, Annu. Rev. Immunol., 11:729-766 (1993) and A. Lanzacecchia, J. Exp. Med., 181:1945-1948 (1995)]. Upon over-expression of the protein or upon alteration of its processing, a sufficient amount of these low avidity epitopes can associate with MHC determinants and, provided that presentation is mediated

by professional antigen presenting cells (APCs) within the context of lymphatic tissue, result in the induction of a T cell-mediated immune response.

Peptides delineated from a mutated region of p53 were shown to induce in experimental animals a T cell-mediated immune response to tumors expressing the homologous mutation [M. Yanuck et al, Cancer Res., 53:3257-3261 (1993)]. Thus, point mutations of self proteins can induce an immune response provided the amino acid exchanges are flanked by anchoring residues that are able to bind to MHC determinants [H. Rammensee et al, Ann. Rev. Immunol., 11:213-244 (1993)]. However, the immune response to individual epitopes is genetically restricted, e.g., by MHC, thus limiting the usefulness of single epitope vaccines.

Recombinant viral vaccines, such as those based on poxviruses (vaccinia, canarypox or avipox) and adenoviruses, have been used for years in experimental animals as well as in clinical trials [T. Wiktor et al, Proc. Natl. Acad. Sci. USA, 81:7194-7198 (1984) and N. Restifo, Curr. Opin. Immunol., 8:658-663 (1996)]. These viral vaccines generally induce potent T and B cell-mediated immune responses against the inserted gene product upon a single inoculation and the recombinant viruses are sufficiently attenuated to allow for their use in humans with acceptable side effects. A clear disadvantage of viral recombinant vaccines is the potent immune response against the antigens of the vaccine carrier that can overshadow the response to the insert. The immune responses to the antigens of the viral recombinant might be advantageous by providing additional helper T cells. However, the presence of these antigens can prevent the use of the homologous construct for subsequent booster immunizations by inducing neutralizing antibodies.

One of the latest additions to the field of vaccinology is the DNA vaccine, which was first described in 1992 [D. Tang et al, Nature, 356:152-154 (1992)]. DNA vaccines have advantages and disadvantages over more traditional types of vaccines. One of the primary advantages is the ease with which DNA vaccines can be constructed and manipulated. Immunologically, these vaccines seem to provide their own adjuvant in the form of CpG sequences present in the bacterial backbone [A. Krieg et al, Nature,

374:546-549 (1995) and Y. Sato et al, Science, 273:352-354 (1996)]. Unlike inactivated vaccines, DNA vaccines cause de novo synthesis of proteins in transfected cells leading to association of antigenic peptides with MHC class I determinants and hence to activation of cytolytic T cells. In addition, the kinetics of the immune response upon DNA vaccination differ from that seen upon immunization with a traditional antigen; the immune response in most systems comes up slowly [Z. Xiang et al, Virology, 209:569-579 (1995)] and then stays at plateau levels for a long period of time providing in at least one mammalian system life-long immunity [M. Yakaukas et al, DNA Cell Biol., 12:771-776 (1994)]. This is presumably a reflection of the long-lasting persistence of vectors and vector encoded proteins in cells transfected in situ [J. Wolff et al, Hum. Mol. Genet., 1:363-369 (1992)]. Further, DNA vaccines do not elicit measurable immune responses to the carrier [i.e., the vector DNA], thus allowing for their repeated use.

DNA vaccines presumably induce a potent immune response by causing persistent antigen expression. Mutant p53 in which the mutation is in the hot spot domain may have a dominant/negative effect on wild type p53 thus potentially causing instability in cell cycle control.

Despite all of this knowledge in the art, there remains a need in the art for methods and compositions which are efficacious in inducing immunity to antigens on tumor cells for both the treatment and prophylaxis of cancer. Therefore, a need exists for different mutants that cause complete biological inactivation of the vaccine-delivered p53.

Summary of the Invention

In one aspect, the invention provides a pharmaceutical or vaccine composition comprising a recombinant vector comprising a p53 gene encoding a biologically inactive p53 product (e.g., murine p53 mutated at amino acid 338 or a comparable mutation with identical biological consequences for p53 derived from other species) and a suitable pharmaceutical carrier, and optionally containing a selected

chemokine, cytokine or combinations thereof. In this vector, the p53 gene is under the regulatory control of a promoter which directs the expression of the inactive p53 gene product in a mammalian subject. The vector is capable of delivering the gene to said subject without becoming toxic thereto,

In another aspect, the invention provides a pharmaceutical or vaccine composition comprising a DNA vector which induces an immune response to a tumor cell. This DNA vector encodes a biologically inactive p53 gene product suitable for induction of an immune response under the regulatory control of a promoter which directs the expression of the gene product in a mammalian subject, and a suitable pharmaceutical carrier. Optionally, a chemokine, cytokine or combinations thereof in the form of naked DNA, or in a plasmid vector may form part of this composition.

In still a further aspect, the invention provides a recombinant vector comprising a p53 gene fused to a signal sequence under the regulatory control of a promoter which directs the expression of the fusion sequence in a mammalian subject. The signal sequence retargets the p53 gene away from the nucleus toward a pathway suitable for association with MHC class I or II determinants.

In yet another aspect, the invention provides a pharmaceutical or vaccine composition comprising the above-described recombinant vector in a suitable pharmaceutical carrier, with an optional chemokine, cytokine or combinations of several adjuvants.

In yet another aspect, the invention provides a method for inducing an immune response to a tumor cell in a mammal comprising the step of administering to the mammal (a) an above-described recombinant vector comprising a mutant p53 gene encoding a biologically inactive p53 product, and (b) an optional suitable amount of a chemokine, cytokine or combinations thereof.

In still a further aspect, the invention provides a method of inducing an immune response to a tumor cell in a mammal comprising the step of administering to a subject a pharmaceutical or vaccine composition comprising (a) a DNA molecule which by

encoding a biologically inactive p53 gene product under the regulatory control of a promoter which directs the expression of said gene product in a mammalian subject, and (b) an optional suitable amount of a chemokine, cytokine or combinations thereof, induces an efficacious immune response to a tumor cell.

In yet another aspect, the invention provides a method for inducing an immune response to a tumor cell in a mammal comprising the step of administering to the mammal (a) an above-described recombinant vector comprising a p53 gene fused to a signal sequence; and (b) an optional suitable amount of a chemokine, cytokine or combinations thereof.

In still a further aspect, the invention provides a method of inducing an immune response to a tumor cell in a mammal comprising the step of administering to a subject a pharmaceutical or vaccine composition comprising (a) a DNA molecule encoding a p53 protein fused to a signal sequence under the regulatory control of a promoter which directs the expression of the gene product in a mammalian subject and (b) an optional suitable amount of a chemokine, cytokine or combinations thereof, induces an immune response to a tumor cell.

Still another aspect of this invention is a method for inducing an immune response to a tumor cell comprising the steps of (a) first administering to a subject a suitable amount of a DNA sequence encoding a p53 antigen, such as the DNA compositions described above; and (b) subsequently boosting the subject with a suitable amount of a recombinant vector comprising a p53 gene as described above. Optionally this method may include administration of a chemokine, cytokine or combinations thereof.

In yet another aspect, the invention provides a method for inducing an immune response to a tumor cell in a mammal comprising the step of co-administering to said mammal: (a) a recombinant vector comprising a p53 gene under the regulatory control of a promoter which directs the expression of said gene product, and (b) an optional suitable amount of a chemokine, cytokine or combinations thereof that enhances an immune response.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a graph showing percentage of tumor-free mice vs. Days after first and second tumor challenge for mice administered a vaccinia virus carrying wildtype murine p53 (Vp53-wt) and challenged with (A) 2 x10^5 or (B) 1 or 5 x 10^6 GL261 tumor cells (solid square); mice administered the control construct, an inactivated vaccinia virus carrying a rabies glycoprotein gene (VRG) (open squares); mice administered the VRG construct and receiving a challenge of 2 x 10^5 GL261 cells (A, white squares) or 1 x 10^6 GL261 cells (B, cross-hatched squares) or 5 x 10^6 GL261 cells (B, solid squares). Lines for both second challenge doses of the Vp53-wt vaccinated mice overlap.

Fig. 2A is a graph showing the % of peroxidase stained area/total tissue area of thin solid tissue GL261 tumor sections from VRG-vaccinated mice (white bars) or Vp53-wt vaccinated mice (black bars) analyzed for inflammatory infiltrate by staining for cell surface markers or for cytokines on T helper cells (CD4), cytolytic T cells (CD8), B cells (B220), granulocytes, macrophages (mac-1), and activated APCs (B7.2) with antisera or monoclonal antibodies. Cytoplasmatic expression of IFN-γ, IL-4, and TNF-α is also shown. Two to three representative sections were analyzed and the means of the obtained values are shown.

Fig. 2B is a graph similar to that of Fig. 1A but representing necrotic areas of the tissue sections having a more discrete infiltrate.

Fig. 2C is a graph similar to that of Fig. 1A, but representing the interphase between solid and necrotic parts of the tissue which showed the most pronounced aggregation of infiltrating cells.

Fig. 3 is a graph depicting the results of a group of C57Bl/6 mice first inoculated with 1 TD_{100} of GL261 tumor cells and, subsequent to the appearance of visible tumors, vaccinated with the Vp53-wt vaccine. The following day IL- 12 treatment was

initiated for 5 days at 0.25 µg/mouse. Percentage of surviving mice is reported vs. day following IL-12 treatment. The symbols represent three separate groups of mice. The symbols are: groups with small tumors (cross-hatched square); groups with large tumor requiring euthanasia (white square); and groups with no tumors, i.e., with complete regression (black square).

Detailed Description of the Invention

The present invention provides methods and compositions for inducing immune responses to tumor cells which over-express p53 or express mutated p53. According to one aspect, this invention involves a therapeutic method for the treatment of existing cancers and/or a prophylactic regimen for the prevention of cancers, typically in patients at high risk for the disease. The compositions useful in these methods include recombinant virus vectors and DNA 'vaccine' compositions, e.g., expression vectors or naked DNA, containing species homologous p53 genes. Methods of administering these compositions, optionally with one or more selected chemokine, cytokine or combinations thereofs, induce an anti-tumor immune response. The methods of the invention have proven effective in murine animal models of cancer, both by pre-vaccination prior to challenge with a tumor cell and in an animal model which had existing tumors prior to administration of the vector constructs of this invention.

I. The p53 Antigen

The sequences for human p53 are known and reproduced herein as SEQ ID NOS: 1 and 2. Murine p53 sequences are reported in GenBank [see, also, Matlashewski et al, EMBO J., 13:3257-3262 (1984) and International Patent Application No. WO94/02167]. The murine amino acid sequence is also reported in SEQ ID NO: 4. The p53 sequences for other mammal species has been described [Soussi et al, Oncogene, 5:945-952 (1990)].

The various regions of p53 have been described. For example, the ability of p53 to bind DNA has been mapped to a region with amino acids 90-290 of p53 [Halazonetis and Kandil, EMBO J., 12:5057-5064 (1993)]; the p53 transactivation domain has been mapped to within amino acid residues 1-90 [Fields et al, Science, 249:1046-1049 (1990)]; the oligomerization domain maps to within residues 322-355 [Wang et al, Mol. Cell. Biol., 14:5182-5191 (1994)]; and regulation of DNA binding maps to within amino acid residues 364-393 of human p53 [SEQ ID NO: 2] or to corresponding region of mouse p53 [SEQ ID NO: 4].

A. Biologically active p53

Because biologically active p53 causes apoptotic cell death upon overexpression, it is unsuitable for incorporation into a composition or vaccine for which the efficacy depends on the production of antigen for a relatively long time by transfected or infected mammalian cells. Thus, in the methods or compositions of this invention described in detail below, the biologically active p53 is only used as the p53 antigen for those recombinant virus vectors which are inherently toxic to the infected cell prior to apoptosis caused by overexpression of the p53 gene product. The full-length biologically active p53 is not appropriate for the below-described DNA 'vaccine' compositions and for recombinant viruses which are potentially able to chronically infect the mammalian subject and caused sustain gene expression.

In a particularly desirable embodiment, the biologically active p53 is wildtype murine full-length cDNA coding sequence of p53. For use in humans, the nucleotide and amino acid sequences of human p53 are desirable. Similarly for use in other mammalian species, the corresponding mammalian p53 homolog sequences are used. Other useful biologically active p53 sequences are known and include such modified p53 proteins as those described in U. S. Patent No. 5,573,925, among others.

B. Biologically inactive forms of p53 protein

Preferred for use in the DNA vaccine compositions and for recombinant viruses capable of long-term expression are mutant or modified p53 genes

which encode biologically inactive forms of p53. Among such inactive p53 antigens are those which contain at least one point mutation in the tetramerization domain of the protein, particularly which cause the p53 protein to be unable to functionally bind to corresponding p53 proteins to form biologically active tetramers. One such mutation has been described in murine p53, containing a point mutation at amino acid 338 of murine p53, which prevents tetramerization of the protein and thereby causes functional inactivation [see, e.g., J. Waterman et al, EMBRO J., 14:512-519 (1995)]. Due to a point mutation in the tetramerization domain, p53 can lose the ability to participate in binding to other p53 molecules necessary for biological functions [see, also, U. S. Patent No. 5,573,925, issued November 12, 1996 and incorporated by reference herein]. Similarly, a human p53 may be designed which contains a homologous mutation. Other species' p53 proteins may be similarly made inactive by homologous mutations. It is also anticipated that other modifications to p53 which make it biologically inactive may be included in such sustained recombinant virus compositions, expression compositions or other DNA vaccine compositions.

Other mutant or modified p53 genes for use in these virus recombinant compositions or in the DNA vaccines include mutant murine p53 lacking amino acids 1-70 and corresponding deletions in homologous mammalian p53 sequences.

C. P53 Fusions Which Permit Retargeting of the Antigen In the Cell

Still other embodiments of the p53 antigen useful in the virus vector compositions or DNA vaccine compositions described below include a p53 (preferably a biologically inactive p53 as described above) fused to a signal sequence. By "signal sequence" as used herein is meant any viral, bacterial, fungal, yeast, and other signal sequences known to one of skill in the art or any signal sequence from any secreted mammalian protein. As one example, the adenoviral type 2 signal sequence is employed in the examples. A p53 construct which encodes this fusion product should retarget the p53 antigen to cellular compartments, e.g., the endosomal or lysosomal pathway, which permit

epitope peptides of p53 to associate with MHC I or class II determinants. This fusion of p53 should permit optimal induction of an immune response against the cancer.

This functional characteristic is incorporated into the DNA construct by engineering a p53 protein (including wt p53, or preferably a mutant, such as the biologically inactive form of p53 mutated at aa338 or at a homologous site in another mammalian p53, or a truncate) as a fusion protein linked to a signal sequence. The signal sequence overrides the nuclear localization domain of p53. One such desired signal sequence may be derived from adenovirus human strain 2. Other signal sequences may be selected by one of skill in the art. Such p53 fusion constructs may be used in the recombinant viruses described above or in expression vectors or "naked DNA" compositions described below.

II. Recombinant Virus Compositions of the Invention

According to one embodiment of the present invention, a recombinant viral expression vector is constructed to carry the p53 gene. Virus recombinants which are cytolytic and induce immunity by rapidly expressing high amounts of antigen prior to causing the demise of the infected cells can be employed to express full-length biologically active p53 in the subject to which the recombinant is administered. These recombinants are useful if the toxic effect of the virus upon the cell occurs prior to or concomittantly with the apoptotic effect of p53 overexpression. If the viral recombinant has the potential to cause persistent infection, then a biologically inactive form of the p53 protein is used in these vectors.

A suitable virus for such manipulation may be selected from among many known virus vectors which have been reported to deliver genes to the cells of a mammalian subject. Known virus constructs for delivering genes to a subject include vaccinia virus. For example, an inactivated vaccinia virus carrying a rabies glycoprotein gene (VRG) has been reported [B. Brochier et al, Vaccine, 12:1368-1371 (1994)]. Other vaccinia vectors for other disorders are known. See, e.g., U. S. Patent No. 5,494,807;

U. S. Patent No. 5,494,671; and U. S. Patent No. 5,443,964. As illustrated in the examples below, the Copenhagen vaccinia strain [T. J. Wiktor *et al*, Proc. Natl. Acad. Sci. USA, 81:7194-7198 (1984)] is desirably used in the preparation of recombinant viruses expressing p53 of this invention.

Similarly, adenovirus vectors have been reported as useful to deliver cytomegalovirus genes to subjects for vaccine or treatment of CMV infection. See, e.g., S. Plotkin et al, European Patent Application No. 389,286, published September 26, 1990; Davis, U.S. Patent No. 4,920,309; L. Prevac, J. Infect. Dis., 161:27-30 (1990); T. Ragot et al, J. Gen. Virol., 74:501-507 (1993); M. Eliot et al, J. Gen. Virol., 71:2425-2431 (1990); and S. C. Jacobs et al, J. Virol., 66:2086-2095 (1992); and Z. Xiang et al, Virology, 219(1):220-227 (1996)]. Thus, adenovirus constructs, among which many have been reported, are useful as the recombinant virus constructs for delivery of the p53 wildtype gene.

Still other virus constructs which may be used in the practice of this invention include retrovirus vectors [J. Tartaglia et al, AIDS Research and Human Retroviruses, 9(Suppl. 1):S27 (1993)], canarypox vectors [W. Cox et al, Virology, 195(2):845-850 (1993) and J. Tartaglia et al, J. Virol., 67(4):2370-2375 (1993)], as well as other poxviruses and insect viruses, such as baculovirus. The selection of the particular virus vector useful to carry the p53 gene is not a limitation upon this invention.

In the recombinant virus of the invention, the p53 sequence may be under the control of the selected promoter. By "promoter" is meant a DNA sequence which may be homologous or heterologous to the virus vector, and which functions to regulate and direct the expression of the p53 gene product in a mammalian cell. This invention is not limited by the selection of the promoter useful in the vector construct, i.e., a native viral promoter is generally replaced using techniques known to those of skill in the art. Desirable promoters include the CMV promoter, the Rous sarcoma virus LTR promoter/enhancer, the SV40 promoter, and the chicken cytoplasmic ß-actin promoter [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. Still other promoter/enhancer

sequences known in the art, including naturally occurring viral promoters or mammalian promoters, may be readily selected by one of skill in the art. These vectors may contain other suitable regulatory sequences. These sequences are known to and readily available to the skilled artisan.

The selected p53 gene sequence may be inserted in any region of the recombinant viral genome not essential for the formation of the recombinant virus. For example, if the virus is vaccinia, the gene may be inserted into the thymidine kinase (tk) region; if the virus is adenovirus, the gene may be inserted into the E1 region. If the virus is canarypox, the virus may be inserted into a similarly non-essential region. Such regions may be selected by one of skill in the art. Conventional genetic engineering techniques are employed to prepare the virus recombinants. See, for example, Sambrook *et al*, "Molecular Cloning. A Laboratory Manual", 2d edition, Cold Spring Harbor Laboratories, NY (1989).

III. The DNA Compositions of the Invention

In an alternate embodiment of this invention, the p53 composition comprises a DNA sequence encoding a p53 antigen without a viral carrier. These DNA compositions may be in the form of non-viral expression vectors or may consist of only a DNA sequence containing a promoter and the selected p53 gene. The efficacy of these DNA vaccines depends on the production of antigen for a relatively long time by transfected cells.

Examples 3 and 4 disclose various illustrative polynucleotide (DNA) compositions including either full-length wild-type p53, or a truncated p53 (deletion of amino acid residues 1-70 of the transactivation domain), or a mutant p53 (e.g., containing a single point mutation in position 135 of p53, or containing double point mutations in positions 168 and 234 of p53).

However, an improved DNA vaccine composition contains the mutant p53 gene encoding a biologically inactive p53. The illustrative DNA vaccine construct of Example 4A expresses p53 with a point mutation at position 338 in the tetramerization domain which prevents tetramerization of the protein and thereby causes functional inactivation [J. Waterman et al, EMBRO J., 14:512-519 (1995)]. Similar mutant p53 may be made using homologous mutations of the human p53, or other mammalian p53. Still an alternative and improved DNA construct of the invention permits retargeting of p53, preferably channeling of the p53 antigen towards the endosomal pathway by using the p53-viral sequence fusion described above.

The DNA sequences, together with nucleotide sequences encoding appropriate promoter sequences and regulatory sequences ("naked DNA"), may be employed directly as a therapeutic composition according to this invention [See, e.g., J. Cohen, Science, 259:1691-1692 (March 19, 1993); E. Fynan et al, Proc. Natl. Acad. Sci., 90: 11478-11482 (Dec. 1993); J. A. Wolff et al, Biotechniques, 11:474-485 (1991); International Patent Application PCT WO94/01139, published January 20, 1994, which describe similar uses of 'naked DNA', all incorporated by reference herein.

Briefly, the DNA encoding the preferably biologically inactive p53 protein or a mutant or truncate thereof is inserted into a nucleic acid cassette. This cassette may be engineered to contain, in addition to the p53 sequence to be expressed, other optional flanking sequences which enable its insertion into a vector. This cassette may then optionally be inserted into an appropriate DNA plasmid or expression vector downstream of a promoter, an mRNA leader sequence, an initiation site and other regulatory sequences capable of directing the replication and expression of the p53 sequence *in vivo*. This vector permits transfection of subject's cells and expression of the p53 protein *in vivo*.

Numerous types of appropriate expression vectors are known in the art for mammalian (including human) protein expression, by standard molecular biology techniques. Such vectors may be selected from among conventional vector types including bacterial, e.g., *E. coli*-based vectors, fungal vectors, yeast or phage expression

vectors. Other appropriate expression vectors, of which numerous types are known in the art, can also be used for this purpose.

Methods for obtaining such expression vectors are well-known. See, Sambrook et al, Molecular Cloning. A Laboratory Manual, 2d edition, Cold Spring Harbor Laboratory, New York (1989); Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

IV. Methods of Treatment/Prevention Using the Compositions of the Invention

A. Recombinant Virus and Optional Chemokine, cytokine or combinations thereof

A recombinant virus bearing a gene encoding a p53 protein may be administered to a human or veterinary patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle is sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

Optionally, a composition of the invention may be formulated to contain other components, including, e.g. adjuvants, stabilizers, pH adjusters, preservatives and the like. Such components are well known to those of skill in the pharmaceutical art.

The recombinant viruses are administered in an "effective amount", that is, an amount of recombinant virus that is effective in a route of administration to infect the desired cells and provide sufficient levels of expression of the p53 gene product to provide a therapeutic benefit, i.e., protective immunity or tumor regression.

Conventional and pharmaceutically acceptable routes of administration include intranasal, intramuscular, subcutaneous, intradermal, rectal, oral and other parenteral routes of administration. Routes of administration may be combined,

if desired, or adjusted depending upon the immunogen or the disease. The route of administration primarily will depend on the location and nature of the tumor being treated.

Doses or effective amounts of the recombinant replication defective virus will depend primarily on factors such as the type of tumor, the age, weight and health of the subject, and may thus vary among animal or human subjects. For example, a prophylactically effective amount or dose of a virus vector composition according to this invention is that amount effective to induce a protective immune response without seriously negatively threatening the health of the subject. An example of a suitable dose is in the range of from about 100 μl to about 5 ml of solution containing about 1×10^4 to 1×10^{13}, and preferably 1×10^6 to 1×10^9, plaque forming units (pfu)/ml. Other suitable dosages can be selected by the attending physician. The levels of immunity can be monitored to determine the need, if any, for boosters, by T cell proliferation assays, by cytokine release assays or by assays designed to test for cytolytic T cell activity.

Preferably such a recombinant virus composition is also formulated with a chemokine, cytokine or combinations thereof. As an optional, but preferred, component of the methods of this invention, the p53 recombinant vector compositions are co-administered with a selected chemokine, cytokine or combinations thereof. Co-administration includes administration of the chemokine, cytokine or combinations thereof before, contemporaneously with or after administration of the vector. Among such chemokine, cytokine or combinations thereof are included GM-CSF, Interleukin-1 through Interleukin-18, Interferon α, β, or γ, tumor necrosis factor-α (TNF-α), and T cell growth factor-β (TGF-β). Other known growth factors, chemokine, cytokine or combinations thereof and lymphokines, which are commercially available or available from universities and research institutes may also be used for this purpose. One such exemplary cytokine is Interleukin-12 (IL-12), a heterodimeric cytokine. The expression and isolation of IL-12 protein in recombinant host cells is described in detail in International Patent Application WO90/05147, published May 17, 1990 incorporated by reference herein. Recombinant

human and murine IL-12 are also available from Genetics Institute, Inc., Cambridge, Massachusetts, and other commercial and academic sources.

When the method of administering involves co-administration of a chemokine, cytokine or combinations thereof, the effective amount of the cytokine, e.g., IL-12 protein, may vary depending on the factors discussed above, the amount of recombinant virus administered, and the mode of administration. An example of an effective amount of the chemokine, cytokine or combinations thereof is about 0.1 μg to about 0.5 mg of protein per about 25 μg of p53. The co-stimulatory amount can be readily determined by the attending physician depending on the factors identified above.

Desirably, the chemokine, cytokine or combinations thereof is administered by the same route as the virus vector composition. When the chemokine, cytokine or combinations thereof is administered separately from the wt p53 virus vector composition, the chemokine, cytokine or combinations thereof is desirably in the presence of a suitable carrier, such as saline and optionally conventional pharmaceutical agents enabling gradual release of the protein.

As illustrated in detail in the examples below, a number of different compositions expressing mutated or wild-type p53 were constructed to test their ability to induce immune responses in mice which limit the spread of tumor cells carrying a p53 mutation or overexpressing p53. Different prototypes based on recombinant viruses were generated to express wild-type or mutant mouse p53.

It has been surprisingly determined that according to the present invention a recombinant vaccinia virus vaccine expressing wild-type mouse p53 induces in mice a T cell-mediated immune response that provides partial protection to a subsequent challenge with spontaneously arisen, transplantable tumor cells also expressing wild-type p53. As detailed below, the additional treatment of the subject with chemokine, cytokine or combinations thereof, e.g., IL-12, used as a co-stimulant protein administered after challenge with tumor cells provides enhanced protection beyond that which would be

expected by administration of either the recombinant virus or the chemokine, cytokine or combinations thereof alone.

Further, combination treatment composed of the viral recombinant expressing wtp53 and IL-12 protein results in regression of already established tumors. Thus, a method of this invention involving administering the recombinant virus expressing wtp53 with a soluble chemokine, cytokine or combinations thereof had a synergistic effect in inducing protection to tumor challenge and regression of pre-existing tumors.

B. DNA Compositions and Optional Genetic Adjuvants

An optional, but preferred protocol for administration of the DNA compositions of this invention includes the use of plasmid vectors which encode biologically inactive p53. As adjuvants other plasmid vectors which deliver one or more of the selected chemokine, cytokine or combinations thereof listed above are suitable for co-administration with the DNA compositions. Plasmid vectors for p53 vaccination are preferred because they induce a full spectrum of antigen specific immune responses including cytolytic T cells. Plasmid vectors induce long-lasting immunity needed to prevent late recurrences in cancer patients. They provide their own adjuvant in the form of CpG sequences present in the bacterial backbone. Furthermore, they are able to generate T cell mediated immune responses in non responder haplotypes indicating that they might be particularly suitable to induce T cell responses to non immunodominant (i.e., cryptic) epitopes of a self protein, such as p53. Plasmid vectors containing nucleic acid sequences encoding a selected chemokine, cytokine or combinations thereof, e.g., IL-12, are suitable because the cytokine(s) acts locally, where an antigen is expressed and the immune response is initiated thus avoiding systemic toxicity of the cytokine. Such cytokine expressing vectors are generated by well-known techniques [see, e.g., Sambrook *et al*, cited above]. The nucleic acid sequences, preferably in the form of DNA, may be operably linked to promoter sequences directing its expression *in vivo*.

A chemokine, cytokine or combinations thereof nucleic acid sequence may be operably linked to DNA sequences which also encode the p53. Alternatively, 'naked DNA' encoding the chemokine, cytokine or combinations thereof may be in a separate plasmid. Where present in one or two plasmids, the naked DNA encoding the p53 and/or chemokine, cytokine or combinations thereof, upon introduction into the host cells, permits the infection of the subject's cells and expression of both the chemokine, cytokine or combinations thereof and the p53 *in vivo* in close proximity.

When administered as DNA, the composition contains the DNA encoding the p53 and a suitable carrier, such as saline. The chemokine, cytokine or combinations thereof DNA may be administered similarly. Optionally the composition contains some cationic lipids that enhance uptake of DNA. No other components are necessary for such a DNA composition.

When p53 nucleic acid sequences are employed as 'naked DNA' operatively linked to a selected promoter sequence, the amounts of p53 to be delivered may be selected by the physician depending on the same factors detailed above for determination of the recombinant virus dosage. An example of a suitable amount includes between 1 μg to 1000 μg p53 per dose. The routes of delivery may parallel the recombinant virus delivery described above and may also be determined readily by one of skill in the art.

As detailed below in the following examples, the efficacy of the original DNA compositions vaccine was below that of the recombinant viruses expressing wt p53. However, the p53 protein has a nuclear targeting domain which might interfere with appropriate antigen processing upon DNA vaccination. Thus, the use of DNA compositions comprising a p53 antigen fused to a signal sequence should rechannel the antigen towards cellular compartments that favor association of epitopic peptides with MHC class I [B. Minev et al, Canc. Res., 54:4155-4161 (1994)] or class II determinants [K. Lin et al, Res., 56:21-26 (1996)] and thereby improve the efficacy of the DNA compositions in cancer therapy.

The methods of this invention which involve co-administration of the chemokine, cytokine or combinations thereof, or preferably a plasmid vector carrying it, preferably administer the chemokine, cytokine or combinations thereof together with administration of the p53 DNA. The routes of administration may be the same as for the p53 DNA compositions; dosages of chemokine, cytokine or combinations thereofs may be adjusted to achieve a similar local level achieved by administration of the soluble protein as described above. Also, the chemokine, cytokine or combinations thereof DNA may be administered as part of the therapeutic composition or separately, but contemporaneously with the DNA sequence encoding the wt p53 antigen.

In general, the administration of DNA compositions for cancer immunotherapy induces both CD4+ and CD8+ T cell-mediated immune responses to the expressed tumor antigen. The apparent ability of DNA vaccines to overcome genetic unresponsiveness, a potential problem for cancer vaccines expressing self-antigens, is also advantageous. The long duration of the immune response induced by DNA vaccines is likely to prevent late recurrences of disease in cancer patients caused by dormant tumor cells.

C. Priming and Boosting Methods

Because the rather slow kinetics of the immune response elicited by DNA compositions may be of concern in patients with pre-existing tumors, a combination method consisting of administering a p53 DNA composition of the invention and a viral recombinant of the invention as well as suitable chemokines, cytokines or combinations thereof co-stimulants is anticipated to accelerate and enhance the immune response.

Thus, a further embodiment to the methods of administering the recombinant viruses and DNA compositions of this invention involves augmenting the desired immune response by using priming and boosting steps. According to this method, the above-described DNA compositions are first administered to the mammalian subject in the dosages disclosed above to "prime" the subject's immune system. Subsequently, a

recombinant virus expressing p53 as described above is administered as a "booster" in a suitable dosage, also as described above in Part A.

A method involving priming and boostering alone is anticipated to enhance the therapeutic and prophylactic effects of the compositions of this invention. However, an optional step in the priming and boosting method involves co-administering chemokine, cytokine or combinations thereof co-stimulants in the form of plasmid vectors. For DNA compositions carrying p53, the effect of chemokine, cytokine or combinations thereofs can be localized by co-inoculating a p53 antigen-encoding DNA in a plasmid vector with a plasmid encoding a chemokine or cytokine, or several plasmids encoding combinations thereof. This method is anticipated to further augment the efficacy of DNA compositions to the tumor-associated p53 antigen.

The following examples illustrate the components and method of this invention, and do not limit the scope of the present invention, which is embodied in the appended claims.

Example 1 - Cell Lines

A. Tumor Cell Lines

A number of spontaneously arisen, transplantable cell lines that express endogenously either high levels of wild-type or mutant p53 are used as tumor targets. These tumor cell lines are derived from two different mouse strains, are representative of common human types of cancer, and vary in a number of parameters likely to affect immune responses. The tumor cell lines (murine and human) were obtained and/or generated by conventional techniques. From the tumor cells, transcripts of p53 were amplified by reverse transcription polymerase chain reaction (RT-PCR) and the PCR products were sequenced to characterize potential mutations in the hot spot region of p53 (See, Table 1). In addition, for some of the cell lines the levels of p53 expression were established by indirect immunofluorescence or by Western Blot analysis using a p53 specific monoclonal antibody (Ab-l, Oncogene, Cambridge, MA) or a

monoclonal IgM antibody which recognizes a linear epitope of the C terminus of p53 as determined by Western Blot analysis. This latter antibody was generated by using synthetic peptides coupled to a T helper cell epitope in collaboration with Dr. L Otvos (The Wistar Institute), and termed mAb-18 [R. Hoffmann et al, Peptides: Chemistry, Structure and Biology, eds. J. Tam et al, Kluwer, in press (1997)].

All of the tumor cell lines that were positive for p53 expressed substantially higher amounts of p53 compared to primary fibroblasts.

Cell lines were titrated in syngeneic mice upon s.c. inoculation of graded numbers of cells to establish the minimal tumorigenic dose (TD_{100}), defined as the number of cells that cause visible tumors in 100% of control mice within 2-4 weeks. Some of the cell lines were characterized for secretion of TGF-ß [G. Bellone et al, J. Cell. Phys., 172:1-11 (1997)], IL-3/GM-CSF using an indicator cell system [B. Kreider et al, Mol. Cell. Biol., 10:4846-4853 (1990)] or IL-10 by an ELISA. These cell lines were also tested for expression of MHC class I determinants by indirect immunofluorescence using a monoclonal antibody reactive to D^b and L^d followed by FACS analysis. The results of experiments to determine expression of MHC class II determinants are summarized in Table 1. The symbols used in the table include "wt" for wildtype p53; "mu" for mutant p53, which in MethA-34 contains double mutations in position 168 (Glu to Gly) and 234 (Met to Ile); "TD_{100}" defined above.

Table 1

Tumor Line	Tumor Type/Origin	p53 Type/ Level	1 TD_{100}	TGF-β secretion Active/ total (pg/ml)	GM-CSF secretion	MHC CI1 expression
GL261	Glioma/C57Bl/6	wt/++	$1x10^5$	70/936	+/-	+
Bl6.F10	Melanoma/ C57Bl/6	wt/n.t.	$5x10^4$	14/176	nt	+
CT-26	Colo-Rectal CA/Balb/c	wt/++	$5x10^4$	1/2075	+	+
MethA-34	Fibrosarcoma/ Balb/c	mu/++++	$1x10^6$	n.t.	-	++
66.1	Mammary CA/Balb/c	wt/-	$4x10^4$	27/759	-	-
410.4	Mammary CA/Balb/c	wt/++	$3x10^4$	51/3352	+++	+
t(10)lras E7E6	Transformed/ Balb/c Fibroblasts	none	$2x10^4$	n.t.	+++	-

The tumors listed in Table 1 above are spontaneously arisen, transplantable tumors of non-viral etiology (including the mammary carcinoma lines which are not caused by murine retroviruses), which are representative of common types of human cancers. They express high levels of p53, secrete different types and levels of chemokine, cytokine or combinations thereofs, and vary in the amount of cell surface expression of MHC class I molecules, all of which are parameters that may have an effect on the ability of the vaccine induced immune effector mechanisms to limit tumor growth.

B. Fibroblast cell line (10)1

A fibroblast line, termed (10)1, immortalized due to the spontaneous loss of both p53 alleles [M. Harvey et al, Genes Dev., 5:2375-2385 (1991)] was transformed by a triple promoter retroviral vector [R. Overell et al, Mol. Cell. Biol., 8:1803-1808 (1988)] expressing v-Ha-ras under the control of the long terminal repeat, E7, of HPV-16 under the control of the HSV promoter and E6 of HPV-16 under the control of the SV40 promoter. Transformed cells, termed t(10)1rasE7E6, were selected *in vivo* by passage in nude Balb/c mice [Jackson Laboratory, Bar Harbor, ME]. This tumor cell line, which lacks p53 expression, is used as a control.

C. p53 Negative cell line

A p53 negative cell line of the C57Bl/6 H-2^b haplotype is generated by using the same approach that resulted in the (10)1 cell line. Alternatively, such a cell line is generated by chemically inducing a tumor cell line in p53 knock-out (p53KO) mice (backcrossed to C57Bl/6) [Jackson Laboratory, Bar Harbor, ME] or by transforming embryonic fibroblasts from p53KO mice with 2 viral oncogenes.

A primary fibroblast cell line from C57Bl/6 embryos is established and maintained in culture to generate p53 loss mutants. After several months in culture the line is subcloned. Colonies are expanded and tested for p53 transcripts by reverse transcription polymerase chain reaction (RT-PCR). A limited number of colonies that failed to show the p53 specific message are stained for p53 using the Ab-1 (Oncogene).

In the event that a p53 loss mutant is not generated by prolonged culture of C57Bl/6 embryonal fibroblasts, tumors are chemically induced in p53KO mice backcrossed onto the C57Bl/6 background. Interbreeding of p53KO heterozygote mice (Jackson Laboratories, Bar Harbor, MN) produces p53KO homozygote pups. Homozygote p53KO mice are inoculated with MethA at 2 mg in 100 μl of lard oil per mouse given s.c. The tumors, which generally develop 2-3 months later (at least in C57Bl/6 mice), are excised and a cell line established and tested for p53 expression

(RT-PCR) and expression of MHC class I determinants (indirect immunofluorescence followed by FACS analysis).

In case the MethA induced tumors lack MHC class I expression, an embryonal fibroblast line generated from p53KO mice is transformed with a double promoter retroviral vector (already available and tested for its ability to transform fibroblasts) expressing v-Ha-ras and v-myc. Transformed cells are selected in RAG-2 mice.

Example 2 - Recombinant Viruses

A number of recombinant viruses were generated by conventional genetic engineering techniques. Briefly, three different p53 encoding sequences were employed, resulting in three different p53 proteins:

(a) wild-type p53,

(b) p53 with a single mutation at position 135 (Phe to Ala) in p53 ($p53mu_{135}$), and

(c) p53 with a double mutation, one at position 168 (Glu to Gly), and one at position 234 (Met to Ile) in p53 ($p53mu_{168.234}$).

Vp53-wt (Vacp53-wt) is a recombinant vaccinia virus expressing the full-length wild-type p53. It was prepared as follows: The DNA fragment encoding murine full length wild-type p53 (p53-wt) was excised from a pGEM [Promega, Madison, WI] vector containing murine p53 using restriction sites EcoRI and Hind III flanking the p53 open reading frame (1171 base pairs) [J. L. Waterman et al, EMBRO J., 14:512-519 (1995)].

Using blunt end ligation, the insert was cloned into the SalI site of the multiple cloning site of pSCII transfer vector for vaccinia virus [S. Chakrabarti et al, Mol. Cell. Biol., 5:3403-3409 (1985); B. Moss, NIAID]. The construct was characterized by restriction enzyme analysis in order to select plasmid containing p53-wt sequence in a correct orientation with respect to the promoter.

A recombinant vaccinia virus containing the p53-wt sequence under the control of early/late vaccinia virus H6 (TK^-) promoter [M. E. Perkus et al, J. Virol., 63:3829-3836 (1989)] was generated by homologous recombination with vaccinia virus strain Copenhagen. Thymidine kinase negative cells (TK^-) infected with 0.1 pfu of vaccinia virus were transfected with the pSC11 transfer vector containing the p53 encoding cDNA using calcium chloride precipitation. Viral plaques expanded in selective medium as described in Perkus et al were identified by staining for β-galactosidase expression. Recombinant virus, subjected to three rounds of plaque purification, was amplified and titrated on Tk^- cells. The recombinant viruses were initially characterized by PCR of viral DNA using the above described primers for p53 cDNA. Expression of the p53 protein was confirmed by Western blot analysis of p53 negative (10)1 fibroblasts infected with the vaccinia virus recombinant using Ab-1 and Ab-3 monoclonal antibodies specific for mouse wild-type p53 protein (Oncogene, Cambridge, MA), as described [H. Towbin et al, Proc. Natl. Acad. Sci. USA, 76:4350-4354 (1979)]. The vaccinia rabies virus glycoprotein (VRG) recombinant which is also based on the Copenhagen strain of vaccinia virus has been described [T. Wiktor et al, Proc. Natl. Acad. Sci. USA, 75:3938-3945 (1978)].

Using similar techniques, the p53 mutant genes were cloned into transfer vectors. The expression vectors were tested by restriction enzyme analysis.

Vaccinia virus strain Copenhagen recombinants were generated by homologous recombination in thymidine kinase deficient (Tk^-) cells. The resulting viruses were Vp53-mu_{135} and Vp53$mu_{168.234}$. Vaccinia virus p53 recombinants were expanded and titrated on HeLa or Tk^- cells.

Using similar techniques a recombinant baculovirus expressing wild-type mouse p53 upon infection of insect cells, as determined by Western Blot analysis, was also produced.

All of the viral recombinants were purified by a plaque assay 2-3 times to ensure preparation of stock virus free of wild-type virus contamination. The recombinant viruses were initially identified upon infection of p53 negative cells for expression of p53 transcripts by RT-PCR using appropriate p53 primers and for protein expression by Western Blot analysis.

Example 3 - DNA constructs

A number of DNA constructs were generated using different plasmid vectors with the SV40 or the CMV promoter. For example, one such vector is the high expressing vector pVR1012.2 (Vical, Inc.) in which the kanamycin resistance gene was replaced with the ampicillin resistance gene which contains additional immunostimulatory CpG sequences.

These vectors were designed to express either full-length wild-type p53, or truncated mouse p53 (deletion of aa 1-70 of the transactivation domain), or mutant p53 (using the same mutations that were used for construction of vaccinia virus recombinants of Example 2, i.e., in $p53mu_{135}$ and $p53mu_{168.234}$). Vectors were analyzed by restriction enzymes digestion.

Example 4 - Constructs Containing a Chimeric Biologically Inactive p53-Coupled to a Signal Sequence

A. *pVR1012sigp53mu$_{338}$*

A pGEM type plasmid which carries p53 with a point mutation at position 338 which prevents tetramerization of the protein and thereby renders it functionally inactive [J. L. Waterman et al, EMBRO J., 14:512-519 (1995)], and a pSC11 vector (Thomas Jefferson University) which carries the signal sequence of adenovirus human strain 2, are used to prepare a p53 fusion molecule with the Ad2 signal sequence using conventional genetic engineering techniques.

Briefly described, oligonucleotide primers contain 5' and 3' terminal restriction enzyme sites to amplify the p53 sequence. The PCR product upon restriction enzyme digest is cloned into the pSC11 vector just 3' of the signal sequence. The fusion gene is excised from the pSC11 vector and inserted into the multicloning site of the pVR1012 vector. The original pVR1012 vector constructed for optimized expression of proteins under the control of the CMV promoter contains the kanamycin resistance gene. The kanamycin resistance gene is replaced with the ampicillin resistance gene which contains 2 palindromic AACGTT [SEQ ID NO: 3] sequences which have been described to provide an adjuvant effect to DNA vaccines [A. Krieg et al, cited above; B. Minev et al, cited above].

The resulting construct upon initial testing by restriction enzyme digest is sequenced to ensure in frame insertion and faithful PCR amplification of the p53 gene. The construct is stably transfected into (10)1 cells using pSV2neo for co-transfection and G418 for selection. Stable transfectants are analyzed by indirect immunofluorescence and confocal microscopy to determine localization of the protein using mAb-1 and by Western Blot using mAb-18.

B. Adsigp53mu₃₃₈

An E1-deleted adenoviral recombinant expressing a fusion p53 molecule is constructed using previously described methods [Y. Yang et al, Immunity, 1:433-442 (1994) and Z. Xiang et al, Virology, 219:220-227 (1996)].

Briefly described, the biologically inactive form of mouse p53 (mutation at 338) is constructed as a fusion protein with the mouse GM-CSF leader sequence in order to avoid potential recombination of the adenoviral leader sequence described above. The fusion gene is cloned into the NotI site of the pAdCMV transfer vector by blunt-end ligation, and is sequenced. The transfer vector is linearized with EcoRI and co-transfected with purified adenoviral DNA cut with ClaI to remove the left end of the viral genome into E1 expressing 293 cells [F. Graham et al, J. Gen. Virol., 36:59-72 (1977)]. Cells are overlaid with agarose, viral plaques are selected and screened

for the presence of the p53 sequence (PCR), and then resubcloned twice more to obtain recombinant virus free of wildtype virus contamination.

Once a plaque purified adenoviral recombinant is generated, expression of the p53 fusion protein is tested by Western Blot upon stable transfection of (10)1 cells. The endosomally targeted form of biologically inactive p53 is expressed for E1-deleted adenoviral recombinants similarly to DNA vaccines. Such E1-deleted adeno-non-viruses are cytopathic and thus able to cause prolonged expression of antigen [Y. Yang et al, cited above].

Example 5 - Cytokine expression vectors

A number of vectors expressing mouse chemokine, cytokine or combinations thereofs under the control of the CMV promoter were generated by conventional techniques and tested *in vitro* for expression of the chemokine, cytokine or combinations thereof upon transient transfection of BHK-21 cells. Vectors expressing the following mouse chemokine, cytokine or combinations thereofs are available: IL-2, IL-4, IL-S, IL-10, IL-12 (both chains expressed by different constructs), IL-13, IFN-γ, IL-18, and GM-CSF.

Using a viral system, IL-4, IL-2 (in some mouse strains), IL-13 (in some mouse strains), IL-12, IL-5, and IL-10 were shown to increase some parameters of the immune response; GM-CSF had the strongest effect in enhancing both B and T cell responses [Z. Xiang et al, cited above], IFN-γ caused a reduction of the immune response [H. Ertl et al, Vaccine, 96:83-86 (1996)].

Example 6 - Induction of protective immune response to vaccinia virus recombinants expressing p53

Most of these studies have been based on the Vp53-wt vaccine expressing full-length wild-type mouse p53. To test if tumor cells with abnormally high levels of wild-type p53 could be treated with a composition containing wt p53, the GL261 cell line

which expresses wt p53 was titrated in C57Bl/6 mice. An initial tumor burden of 2 - 5 x 10^5 cells was given subcutaneously to the mice and was shown to result in clearly visible tumors in >90% of mice within 14 days.

Protection experiments were conducted with the GL261 cell line which expresses wild-type p53, according to the methods described in Roth, J. *et al.*, Proc. Natl. Acad. Sci. USA, 93: 4781-4786 (1996), incorporated herein by reference.

Groups of C57Bl/6 mice received different doses of a vaccinia recombinant virus expressing wild-type p53 (termed Vp53-wt). Recombinant Copenhagen vaccinia virus expressing the glycoprotein of rabies virus (VRG) [T. Wiktor et al, cited above] was used as a negative control for *in vivo* experiments. The doses were 2 x 10^7 pfu or 3 x 10^7 pfu. Control mice were left untreated (none) or were treated with VRG. In one experiment, naive mice were used as a control.

The results of a representative experiment to study protection against tumor challenge after administration of Vp53-wt are shown in Fig. 1. The results show the number of mice developing tumors compared to the number of mice of the experiment over an observation time of 100 days.

Using a fairly low dose of the vaccinia recombinant, 50% complete protection of C57Bl/6 mice with regard to the development of visible GL261 tumors was obtained. In this experiment none of the mice immunized with the control construct were completely protected against the development of tumors. In addition, onset of tumor lesions was delayed in Vp53-wt vaccinated mice that were not completely protected.

Tumor-free mice from the first experiment of Fig. 1 that were completely protected against a challenge with GL261 cells received a subsequent challenge 6 weeks later with a higher dose of tumor cells without any further treatment with recombinant vaccinia virus. All control animals developed tumors after 7-10 days. In contrast, all animals that previously received Vp53-wt remained tumor-free over the observation time of 55 days, thus demonstrating that the combination of the virus vector composition and

subsequent low dose tumor cell challenge had induced immunological memory in these mice. The results of the experiment are shown in Fig. 1.

A series of experiments were performed to evaluate if vaccination with the Vp53-wt vaccine induced protection against other tumors. Table 2 shows the summary of several experiments. Protection to GL261 cells was tested in a total of 31 VRG-immune or 34 Vp53-wt-immune mice. Groups of mice (minimal number 8) were immunized with 2×10^7 plaque forming units (pfu) of VRG or Vp53-wt. Two weeks later, mice were challenged with 1-2 TD_{100} of the different tumor cells identified in the table. Mice that failed to develop tumors within the observation periods (50-100 days) were scored as resistant. For mice that developed tumors, the mean day from the challenge until visible onset of tumor growth (>2 mm in diameter) $\pm$ standard deviation was recorded. Significant of the difference between onset in VRG and Vp53-wt vaccinated mice was calculated by a student T test.

Table 2

Vaccine	Cell Line	% Complete Protection Against Tumor Growth	Onset of Tumors (days ± SD)	Significance
VRG	GL261	10	28.5 ± 15.1	
Vp53-wt	GL261	76	51.3 ± 24.6	0.000001
VRG	B16.F10	0	23.5 ± 4.1	
Vp53-wt	B16.F10	0	30.4 ± 7.8	0.035
VRG	66.1	0	28.0 ± 5.7	
Vp53-wt	66.1	30	45.3 ± 9.1	0.017
VRG	410.4	0	18.9 ± 3.2	
Vp53-wt	410.4	13	24.6 ± 1.5	0.008
VRG	CT-26	0	39.8 ± 1.7	
Vp53/wt	CT-26	10	49.6 ± 5.9	0.003
VRG	t(10)lrasE7E6	0	19.2 ± 2.9	0.038
Vp53-wt	t(10)lrasE7E6	0	16.4 ± 1.7	

Mice immunized with a single dose of 2 x 10^7 plaque forming units (pfu) of the Vp53-wt vaccine were partially protected (i.e., on average 70% complete protection, and delayed onset of tumors in the rest of the mice) against challenge with a minimal tumorigenic dose of the GL261 glioma cell line which caused tumors in 90-100% of the control mice immunized with 2 x 10^7 pfu of the VRG recombinant.

The efficacy of the Vp53-wt vaccine against growth of the GL261 cell line was not enhanced by vaccinating mice with 2 doses of 2 x 10^7 pfu each of the Vp53-wt vaccine (data not shown) which presumably reflects neutralization of the second vaccine

dose by antibodies to surface proteins of vaccinia virus. A similar level of protection to GL261 challenge was achieved with a vaccinia virus recombinant expressing a mouse mutant p53 (Vp53-mu_{135}, data not shown).

No protection could be achieved against the p53-negative tumor cell line, i.e., t(10)1rasE7E6, tested in BALB/c mice. Some protection was obtained against wild-type p53-positive adenocarcinomas such as CT-26 (colon cancer), 66.1 and 410-4 (mammary tumors also tested in BALB/c mice). Protection was not as impressive as that seen in C57Bl/6 mice challenged with GL261 cells; the vaccine induced complete resistance only in a low percentage of mice but resulted in a statistically significant delay of onset of tumor lesions. Lack of complete protection did not correlate with levels of p53 protein expression, for example the 66.1 tumor cell line expresses higher levels of p53 than the GL261 cell line but was more resistant.

Characteristics of the individual tumor cell lines such as density of MHC class I determinants on the cell surface, down-regulation of TAP 1/-2 or LMP2/7, expression of fas-ligand, and secretion of immunomodulatory molecules such as IL-10 or prostaglandin E [P. Musiani et al, cited above] might affect the efficacy of the Vp53-wt vaccine. No correlation was seen between vaccine failures and levels of TGF-ß secretion (Table 1), there was some correlation comparing the two mammary carcinoma cell lines 66.1 and 410-4 between susceptibility to vaccination and levels of MHC expression. Genetic differences between the two mouse strains, i.e., C57Bl/6 and BALB/c, might also influence vaccine induced resistance.

Example 7 - Immune effector mechanisms involved in providing protection to tumor challenge

The immune effector mechanisms that provide protection upon Vp53-wt vaccination were determined in knock-out mice. The following three knock-out mouse strains were used: CD4-KO mice [Z. Xiang et al, Virology, 214:398-404 (1995)] to test for a requirement for T helper cells, ß2 microglobin-KO mice [M. Zijlstra et al, Nature,

344:742-746 (1990)] that lack MHC class I expression and hence CD8+ T cells to test for a putative role of cytolytic T cells and, perforin knock-out mice [C. Walsh et al, Proc. Natl. Acad. Sci. USA, 91:10854-10858 (1994)] to determine a role for cytolysis via the perforin pathway. Results were confirmed and expanded by using in vivo antibody-mediated depletion (see Table 3).

Mice were vaccinated with VRG or Vp53-wt, and were challenged 2 weeks later with GL261 glioma tumor cells. Lymphocyte subsets were depleted by injecting mice I. p. with the appropriate antibody on days -4, -1, +1, +4 and +8 with respect to tumor challenge. The results are shown in Table 3.

Table 3

Vaccine	Recipient	Tumor Free Interval (days ± SD)	% Resistance
VRG	C57Bl/6	28.5 ± 15.1	10
Vp53-wt	C57Bl/6	51.3 ± 24.6	75
Vp53-wt	C57Bl/6-CD4 depleted	14.9 ± 2.6	0
Vp53-wt	CD4KO	22.0 ± 5.5	33
Vp53-wt	C57Bl/6-CD8 depleted	23.1 ± 2.2	0
Vp53-wt	β2m KO	30.1 ± 8.1	3
Vp53-wt	C57Bl/6-NK depleted	20.4 ± 5.4	0
Vp53-wt	perforin-KO	40.0 ± 0	75

Neither CD4KO mice nor ß2m KO mice could be protected by the Vp53-wt vaccine against challenge with the GL261 cell line, indicating that both T cell subsets were needed to limit the growth of the GL261 cell line. Perforin KO mice showed the same level of protection upon vaccination as wild-type C57Bl/6 mice, indicating that perforin-mediated cytolysis was not required for elimination of GL261 tumor cells.

To further ascertain that protection required both CD8+ and CD4+ T cells and to furthermore establish if CD4+ T cells had to be present during the induction phase (i.e., shortly after vaccination) to provide help to CD8+ T cells or if CD4+ T cells were also needed during the effector phase (i.e., at the time of tumor challenge), antibody depletion studies were conducted using the monoclonal antibodies GKI.5 (rat anti CD4) and 53-6-72 (rat anti-CD8). In the initial experiment, mice were treated with the antibodies prior to vaccination. In a subsequent experiment, mice were first vaccinated and then 2 weeks later at the time of tumor challenge treated with the antibodies.

Regardless of the timing, depletion of either CD4+ or CD8+ T cells resulted in loss of protection. In addition, mice that were depleted of either subset showed accelerated growth of tumors which was particularly pronounced after depletion of CD4+ T cells. Natural killer cells were also required for limiting tumor growth in Vp53-wt vaccinated GL261-challenged mice as depletion of this subset by treatment with a rabbit serum to asialo-GM-1 completely abrogated protection.

Example 8 - Histochemical analysis of tumors

GL261 tumors from VRG or Vp53-wt vaccinated mice were analyzed for an inflammatory infiltrate by staining for cell surface markers on T helper cells (CD4), cytolytic T cells (CD8), B cells (B220), granulocytes, macrophages (mac-1), and activated APCs (B7.2) using commercially available antisera or monoclonal antibodies. Frozen sections were stained using the avidin-peroxidase Vectastain Elite BC kit (Vector Laboratories). The sections were developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma) and counterstained with 1% HE. In addition, formalin fixed

sections were stained for cytoplasmatic expression of IFN-γ, IL-4, and TNF-α. Sections were analyzed in a SONY up-5500/5600 microscope with the computational ability to count the number of stained cells per section and to determine the % of peroxidase stained area/total tissue area.

The tumor sections derived from tumors of 1-2 cm in diameter showed three distinct areas: solid tissue which was fairly homogeneously infiltrated with mononuclear cells, necrotic areas with a more discrete infiltrate, and the interphase between solid and necrotic parts of the tissue which showed the most pronounced aggregation of infiltrating cells. Two to three representative sections were analyzed and the means of the obtained values are shown in Figs. 2A-2C. Comparing tumors from VRG immune control mice and Vp53-wt vaccinated mice, the most striking observation was the increased influx of CD8+ T cells into the solid tissue, CD4+ T cells, already present at a high level in tumors of control mice, only increased about 2 fold in the solid tissue. Only a few T cells were scattered throughout the necrotic area or at the interphase, where granulocytes, mac-l+ cells, and B220+ cells were more frequent. B7.2, a co-stimulatory molecule expressed on antigen-presenting cells such as dendritic cells, was mainly found in solid tissue of Vp53-wt vaccinated mice concomitant with CD4+ and CD8+ T cells. Intracytoplasmatic staining for the cytokines, i.e., IFN-γ, IL-4, and TNF-α, showed an increase for all of them, less in solid tissue but mainly at the interphase and in the areas of necrotic lesions. The most pronounced increase was seen for IFN-γ.

Taken together these data indicate that CD4+ T cells play a major role in controlling tumor growth even without specific vaccination. Massive influx of CD8+ T cells is a consequence of Vp53wt vaccination which also enhances (presumably as a bystander effect of antigen specific effector mechanism) recruitment of inflammatory cells such as granulocytes and macrophages. Specific vaccination also increases the influx of chemokine, cytokine or combinations thereof secreting cells, especially those secreting IFN-γ which is a cytokine indicative of a Th1 type immune response.

Example 9 - Combination Treatment with the p53 Composition and IL-12

To enhance vaccine efficacy, a method of administering the p53 recombinant virus combined with the cytokine IL-12 [C. Nastala et al, J. Immunol., 153:1697-1706 (1994); K. Tsung et al, J. Immunol., 158:3359-3365 (1997); and E. Voerst et al, J. Natl. Inst., 87:581-586 (1995)], administered either before or after challenge was performed.

A. In a series of experiments mice were treated s.c. first with the above-described vaccinia recombinant Vacp53-wt or with the VRG rabies construct (2 X 10^7 pfu each composition) as a control. Two weeks later, they were challenged with tumor. At varied times after tumor inoculation, the mice were injected intraperitoneally with 0.25 µg/mouse/day of murine recombinant IL-12 protein for 4 consecutive days.

Groups of 8-10 mice were vaccinated with 2X10^7 pfu of Vp53-wt or VRG virus and were challenged 14 days later with 1 TD_{100} of GL261 cells. Onset of tumor lesions (mean days $\pm$ SD) and % complete protection from tumor development for an observation period of 100 days was recorded. In Experiments 1 and 2 (Table 4) the effect of IL-12 given at different times after challenged was tested. IL-12 was given at 0.25 µg per day ip for 4 days either on days 2-5, 5-9, or 12-15, following challenge. In experiments 3 and 4 (Table 4) the effect of antibody-mediated depletion of lymphocyte subsets on vaccination followed by IL-12 treatment given days 2-5 following challenge was tested. Mice were depleted of CD4 or CD8 T cells or both or of NK cells by injecting the appropriate antibodies on days -4, -1, +1, +4 and +8 with regard to tumor challenge. (n.t. = not tested).

Table 4

			Experiments 1+3		Experiments 2+4	
Vaccine	Depletion of lymphocytes	IL-12, days of treatment	Tumor-free interval (days)	% Protection	Tumor-free interval (days)	% Protection
Vp53-wt	-	-	45 ± 16	63	42 ± 15	42
VRG	-	-	45 ± 24	14	23 ± 4	0
Vp53-wt	-	2-5	68 ± 17	75	34 ± 0	90
VRG	-	2-5	85 ± 7	75	41 ± 13	20
Vp53-wt	-	5-9	>100	100	44 ± 11	30
VRG	-	5-9	42 ± 4	50	41 ± 13	40
Vp53-wt	-	15-19	40 ± 0	63	64 ± 17	10
VRG	-	15-19	50 ± 14	42	35 ± 14	20
Vp53-wt	-	-	40 ± 8	30	54 ± 19	30
VRG	-	-	26 ± 8	0	29 ± 4	0
Vp53-wt	-	2-5	46 ± 0	90	>100	100
VRG	-	2-5	38 ± 16	70	48 ± 0	77
Vp53-wt	CD4	2-5	23 ± 10	10	21 ± 2	0
VRG	CD4	2-5	25 ± 9	0	17 ± 2	0
Vp53-wt	CD8	2-5	27 ± 10	10	43 ± 11	20
VRG	CD8	2-5	22 ± 8	20	26 ± 7	13
Vp53-wt	CD4/8	2-5	n.t.		18 ± 8	0
VRG	CD4/8	2-5	n.t.		16 ± 2	0
Vp53-wt	NK	2-5	n.t.		26 ± 12	0
VRG	NK	2-5	n.t.		38 ± 2	20

IL-12 itself (i.e., in combination with the VRG control vaccine) resulted in partial protection. Nevertheless, a combination of Vp53-wt and IL-12 had a synergistic effect causing complete protection in up to 90-100% of mice. The degree of protection depended on the time lapse between tumor challenge and IL-12 therapy. Best results were obtained when IL-12 was given shortly after challenge.

IL-12 administered in combination with the control vaccine were partially protected if the cytokine was given early, i.e., day 2-5 after tumor challenge. IL-12 given later (i.e., day 5-9) after tumor challenge was more efficacious in mice treated with the p53-expressing virus vector [Brunda, M.J. et al, J. Exp. Med., 178: 1223-1230 (1993).

In groups that were treated with IL-12, 15 or more days post-challenge, protection was markedly reduced. These data indicate that IL-12 alone has an effect on tumor growth if given early, but that better protection is achieved with a combination of IL-12 and a composition containing wt p53.

Depletion of CD4+, CD8+, or NK cells at the time of tumor challenge resulted in a loss of protection, indicating that IL-12 had augmented the vaccine-induced immune response.

B. Groups of mice were vaccinated with 2×10^7 of the Vp53-wt or VRG vaccines. They were challenged 14 days later with 1 TD_{100} of GL261 cells. Some of the groups were treated with IL-12 at 0.25 μg/day i.p. on days 2-5 following challenge. Mice that remained tumor free were rechallenged with an increased dose (i.e. 10 TD_{100}) of GL261 cells or with an unrelated p53-expressing tumor cell line, i.e., B16.F10 melanoma cells. Additional naive mice were also challenged at this time. Data in Table 5 below is reported as mean tumor-free interval after the second challenge in days ± SD and the % of mice that did not develop tumors over the 50 day observation period after the second challenge. The number of mice used for the second challenge is reported as "n". Mice again showed complete protection to challenge with GL261 cells but only partial protection (i.e., mainly delayed onset of lesions) to B16.F10 cells (see Table 5).

Table 5

Vaccine	Il-12	1st Challenge	2nd Challenge	Mean tumor free interval	% complete protection (n)*
Vp53-wt	+	GL261	GL261	>50	100% (9)
VRG	+	GL261	GL261	>50	100% (8)
None	-	-	GL261	18.3 ± 3.2	0% (8)
Vp53-wt	-	GL261	B16.F10	23.3 ± 2.4	22% (9)
Vp53-wt	+	GL261	B16.F10	28.3 ± 4.0	11% (9)
VRG	-	GL261	B16.F10	22.7 ± 2.3	0% (3)
None	-	-	B16.F10	13.7 ± 2.6	0% (9)

Example 10 - Post-Challenge Treatment with p53 Virus Vector and IL-12

Cancer patients are not treated prophylactically but rather after the cancer is sizable enough for diagnosis. Immunotherapy is generally initiated after debulking of the tumor by surgery where possible, followed by conventional therapy. Immunotherapy can have profound effects on the patients' immune system that persist even after removal of the bulk of the cancerous lesion. Tumor cells can affect the immune system at several levels, they can express antigen in association with MHC determinants which can be recognized by naive T cells as the so-called 1st signal. T cells require an additional 2nd signal provided by co-stimulatory molecules such as B7.1 or B7.2, otherwise they are anergized [P. Linsley et al, J. Exp. Med., 173:721-730 (1991)]. Co-stimulatory molecules are expressed by professional APCs but generally not by tumor cells, therefore encounter of naive T cells with tumor cells can cause irreversibly their functional inactivation. Tumor cells can secrete chemokine, cytokine or combinations thereofs such as IL-10,

TGF-ß, or prostaglandin E2 which drives the immune response towards a Th2 pathway or creates an immunosuppressive environment.

This experiment tested if a combination treatment of the p53-carrying vector and IL-12 protein could induce an immune response that causes regression of already established tumors. Groups of C57Bl/6 mice were first inoculated s.c. with 1 TD_{100} of GL261 cells (~ 1.5×10^5 tumor cells). Once the tumors became visible (0.3-0.8 mm in diameter), groups of 10 mice were vaccinated about 30 days later either with 3×10^7 pfu Vp53-wt or with the VRG control construct.

In one experimental protocol, the systemic administration of recombinant IL-12 was initiated 3 days after administration of the p53-expressing viral recombinant by giving 5 intraperitoneal injections of 25 µg of rIL-12 to tumor bearing and Vp53-wt treated mice according to the methods of Nastala, C.L. et al., J. Immunol., 153: 1697-1706 (1994), incorporated by reference herein.

The results are illustrated in Table 6 below, showing the combined therapeutic effect of Vp53-wt and IL-12 given 2 days after the Vp53-wt vaccine on established tumors. In the control group, the progressive growth of tumors was inhibited for some time by the IL-12 treatment. No cures were observed and tumors eventually started growing again, resulting in death of the animals. (Mice with an overly large tumor burden were euthanized for humanitarian reasons). In contrast, in some of the mice immunized with Vp53-wt and treated with IL-12, complete tumor regression was observed.

Table 6

Tumor Cells	Comp'n/pfu	Animals with tumors after IL-12 treatment			% Complete Remission
		1d	30d	40d	
GL261 1.5×10^5	VRG 3×10^7	5/5	5/5	5/5	0
GL261 1.5×10^5	Vacp53-wt 3×10^7	10/10	5/10	5/10	50

Fig. 3 is another example of such experiments showing the kinetics of tumor regression in mice treated with Vp53-wt and IL-12. This method of treatment induces a good protective immune response in contrast with administration of p53 expressing compositions alone or with chemokine, cytokine or combinations thereof alone. Co-administration of chemokine, cytokine or combinations thereof and a wildtype p53 composition produces surprisingly beneficial results. Data clearly show that wild-type p53 present in tumor cells can serve as a target for immunosurveillance. Partial protection to tumor cells expressing p53 is achieved by pre-treatment with a vaccinia recombinant expressing this protein. Protection was improved by additional treatment with IL-12 given early after tumor challenge. The combination of the p53-expressing composition and IL--12 was shown to also result in complete regression of already established tumors.

Example 11 - Characteristics of DNA Vaccines

Each DNA molecule described in Example 3 was tested for induction of protection against a subclone of a MethA tumor cell line expressing the same double mutation of p53 (135/268) as one of the DNA vaccines in Balb/c mice. The DNA vaccines were also tested for induction of protective immunity to other cell lines expressing high levels of wild-type p53, such as the GL261 cells (in C57Bl/6 mice) or the 66.1 and CT-26 cell lines (in Balb/c mice).

In one protocol, Balb/c or C57Bl/6 mice were immunized i.m. with DNA vaccines. They were challenged with 1 TD_{100} of MethA-34 cells or GL261 cells and tumor development was recorded. Table 7 reports the data expressed as % protection which reflects mice that did not develop tumors.

Table 7

Vaccine	Tumor Cell Line	Host	% Protection
pVR1012.2	MethA-34	Balb/c	13
pVR1012.2p53mu$_{168/234}$	MethA-34	Balb/c	50
pVR1012.2	GL261	C57Bl/6	10
pVR1012p53mu$_{168/234}$	GL261	C57Bl/6	30

Results were variable, with some protection achieved against the MethA-34 cell line. There was no significant difference in vaccine efficacy using wild-type or mutant p53 (i.e., a mutation in the mutational hot spot domain). Nor was there any significant difference in vaccine efficacy whether p53 was expressed under the control of the CMV or the SV40 promoter (Table 7 only shows the results for the pVR1012p53mu$_{168/234}$ vaccine and the control construct).

Significant protection could not be induced against other tumor cell lines such as the adenocarcinoma line 66.1 or the CT-26 colorectal carcinoma line (data not shown) indicating that further modifications are needed to improve the efficacy of the DNA vaccine.

Mice that were initially protected by a p53-expressing DNA vaccine to challenge with the MethA-34 cell line as reported in Table 7 and remained tumor-free after the first challenge, were rechallenged 2 months later with 10 TD_{100} of MethA-34 or different tumor cells. Tumor development was reported in Table 8.

Table 8

Mice	2^{nd} Challenge	% Complete Protection
DNA vaccine + MethA-34	MethA-34	100
Naive	MethA-34	0
DNA vaccine + MethA-34	CT-26	0
Naive	CT-26	0
DNA vaccine + MethA-34	t(10)lrasE7E6	0
Naive	t(10)lrasE7E6	0

Mice protected remained completely tumor-free upon rechallenge with a 5 fold higher dose of the same tumor cell line, while an unrelated tumor cell line (i.e., CT-26) or the $p53^-$ t(10)1rasE7E6 cell line caused tumors in 100% of the vaccinated and MethA-34 challenged animals.

The efficacy of DNA vaccines depends on the production of antigen for a relatively long time by transfected cells. However, the efficacy of the DNA vaccines tested was below that of the viral recombinant vaccines. The lower efficacy of the DNA vaccines tested thus far may in part be caused by inappropriate targeting of p53 due to its nuclear localization domain.

Example 12 - Improved DNA Vaccines - Fusion with Signal Sequence

An improved DNA vaccine is constructed as described in Example 4A by channeling the p53 antigen towards the endosomal pathway; i.e., by expressing p53 as a fusion protein linked to a viral signal sequence (i.e., derived from adenovirus human strain 2). The signal sequence overrides the nuclear localization domain of p53, as can be confirmed by immunohistochemical analysis of cells transfected with vectors expressing

the modified p53. The illustrative DNA vaccine construct of Example 4 expresses p53 with a point mutation at position 338 [J. Waterman et al, cited above].

If the nuclear localization domain interferes with endosomal targeting of the chimeric p53, this domain can be removed by site-directed mutagenesis. Upon *in vitro* analysis, the modified DNA vaccine, termed pVRsigp53mu338, will be tested in mice using a vector expressing a biologically inactive form of p53 for comparison and the pVR1012rab.gp vector (expressing the rabies virus glycoprotein) as a negative control. These experiments are conducted with the GL261 tumor system initially in a pre-challenge model and subsequently in a post-challenge model.

Targeting p53 towards the endosomal pathway is expected to enhance the efficacy of the p53 DNA vaccine, i.e., result in a higher degree of protection. Mice are likely to develop an immune response including antibodies, T helper cells, and cytolytic T cells to p53 upon vaccination with the p53 expressing DNA vaccine, which especially for cytolytic T cells is likely higher in mice immunized with a vector expressing an endosomally targeted p53 than in those vaccinated with unmodified p53. Given the type of vaccine used, the immune response is expected to be mixed with Th1 related antibody isotypes (IgG2a) and cytokine secretion pattern (IFN-γ) being predominant over Th2 related responses (IgG1 and IgG2b, IL-4).

The inventor has shown that tumor challenge boosts the immune response, i.e., vaccinated mice that remain tumor free upon challenge with 1 TD_{100} of tumor cells become completely resistant to further challenges with increased doses of tumor cells. Nevertheless, this complete resistance was only observed to challenge with the homologous tumors, challenge with a different p53 expressing tumor caused at best partial protection, i.e., a delay in onset of tumors, indicating that the booster effect by low-dose tumor challenge is not exclusively directed to p53 but broadened to other non cross-reactive tumor-associated antigens. For patients with minimal residual diseases, the vaccine might initiate an immune response which might then be boosted and broadened by the patient's tumor cells.

Example 13 - Combining DNA Vaccines With Chemokine, cytokine or combinations thereofs

Co-inoculation of a chemokine, cytokine or combinations thereof-encoding plasmid vector (i.e., a genetic adjuvant) with a p53 antigen expressing vector is anticipated to result in the simultaneous transfection of cells close to the inoculation site, including professional APCs which upon activation migrate to lymphatic organs initiating the immune response driven by the antigen and influenced by the chemokine, cytokine or combinations thereof.

Using the p53 expressing DNA vaccines of Examples 3 or 4, another method of the invention involves co-administration with plasmid vectors carrying a chemokine, cytokine or combinations thereof such as GM-CSF, IL-4, IL-12, or IL-18, or combinations thereof. Other cytokines or chemokines, which are shown to have a strong adjuvant effect on DNA vaccines may also be employed in this protocol.

Briefly described, mice are inoculated with a mixture of the DNA vaccine and the chemokine, cytokine or combinations thereof expressing vector (~50 μgs is a dose determined to be optimal in mice for most genetic adjuvants in a viral system). Control mice are injected with the p53-expressing vector mixed with an empty plasmid to control for any unspecific effects of the increased load of vector DNA. Mice are boosted with the antigen-expressing construct. Mice are challenged later with tumor cells and tumor development is recorded. Mice that remain tumor-free are challenged later with an increased dose of GL261 cells to establish induction of a long-lasting protective memory response.

Where chemokine, cytokine or combinations thereofs significantly improve vaccine efficacy, the number of booster immunizations are reduced. Alternatively, cocktails of vectors expressing chemokine, cytokine or combinations thereofs that act at different phases of the induction phase of T cells or that act synergistically are employed in this vaccine protocol. For example, a cocktail of GM-CSF, which activates APCs, and

IL-2, which causes proliferation of activated T cells, and IL-12, which causes activation of the innate immune response and promotion of Th1 type immune responses, may form one desirable cocktail. Similarly a combination of IL-12 and IL-18 may be desirable for synergistic activity [H. Okamura et al, Nature, 378:88-91 (1996) and M. Micallef et al, Eur. J. Immunol., 26:1647-1651 (1996)].

For tumor cells that show some, albeit limited, susceptibility to the p53 DNA vaccine, and are able to elicit a recall response in vitro to p53 (either by serving as target cells for p53 specific cytolytic T cells or by inducing lymphocytes from p53-immune mice to secrete chemokine, cytokine or combinations thereofs), chemokine, cytokine or combinations thereofs known to augment the immune response that might be able to limit the growth of a particular tumor are chosen. For example, for tumor cells that are completely resistant to T cell-mediated cytolysis and accordingly fail to recruit CD8+ T cells to tumor lesions in vaccinated mice, but nevertheless re-stimulate a p53 specific T helper cell response in vitro and show an augmented infiltrate with CD4+ T cells and inflammatory cells in vivo, a cytokine, such as IL-4 or GM-CSF, is selected that augments CD4+ T cell responses. For tumors that at least upon treatment with IFN-γ become susceptible to T cell-mediated cytolysis and that histologically show infiltration with CD8+ T cells and upregulation of MHC class I molecules in areas of inflammation, cytokines that enhance cytolytic T cell responses, such as IL-12 or IL- 18 are selected.

The experiments are conducted as described above using a mixture of cytokine-encoding plasmids and the p53 expressing vector for priming. Chemokine, cytokine or combinations thereofs given during vaccination in a pre-challenge model affect the immune response to the vaccine but might not necessarily counterbalance the immunosuppressive environment created by the tumor. A potent vaccine-induced tumor antigen-specific immune response might, provided the antigen is expressed on the tumor cells or the recruited APCs in a form that can be recognized by T cells, override chemokine, cytokine or combinations thereofs that are secreted by the tumor cells and

initiate an inflammatory response that will alter the overall scenario in favor of the immune system.

Chemokine, cytokine or combinations thereofs applied at the time of tumor challenge or later directly into the growing tumor have been shown to effectively tip the balance between tumor growth and anti-tumor immunity [P. Musiani et al, cited above]. The effect of recombinant IL-12 given after challenge on the efficacy of the Vp53-wt vaccine is tested on induction of protective immunity against the different tumor cell lines listed here.

If the IL-12 protein augments efficacy of the viral recombinant to the different tumor cell lines while chemokine, cytokine or combinations thereof adjuvants given in form of expression vectors at the time of DNA vaccination have no or little effect, the IL-12 treatment is included in the vaccine regimen. IL-12, in the form of a recombinant protein, is given to vaccinated mice shortly after tumor challenge as described above, and tumor development is recorded.

Example 14 - Induction of anti-tumor immunity by using prime-boost vaccine regimens

Another vaccine protocol useful in the present invention are a prime-boost protocols using DNA vaccines of this invention for priming and viral recombinants of this invention for booster immunization.

Multiple immunizations with the vaccinia virus recombinant to p53 did not improve vaccine efficacy. The lack of a booster effect was presumably caused by neutralization of the second vaccine dose by antibodies to the carrier. DNA vaccines only induce an immune response to the protein expressed upon transfection of cells; they do not, even after multiple immunizations with high doses of plasmid, induce a measurable sustained response to the carrier (i.e., double stranded DNA, [Z. Xiang et al, cited above]). They are thus highly suitable for prime-boost vaccine regimens [Z. Xiang et al, cited above].

An exemplary prime-boost regimen is as follows: Mice are immunized once with the DNA vaccine and several weeks later they are boosted with the Vp53-wt or the Adsigp53mu$_{338}$ construct. Mice are challenged several weeks after the booster immunization and tumor development is recorded. Prime-boost regimens are anticipated to improve vaccine efficacy. A further modification of this protocol involves DNA priming in the presence of a vector expressing the most efficacious chemokine, cytokine or combinations thereof combined with a booster immunization with the most efficacious viral recombinant. Mice are challenged with tumor cells and analyzed.

Mice with pre-existing tumors are expected to show a change in the immune response to vaccination, either by responding with a more preferential Th2 type response or by demonstrating a comparative lack of responsiveness due to peripheral tolerization or chemokine, cytokine or combinations thereof-mediated suppression of the immune system. Chemokine, cytokine or combinations thereofs are expected to at least in part reverse these effects.

Improving antigen-expression by the DNA vaccine and enhancing the immune responses further by prime-boost immunization is expected to result in better protection (i.e., complete resistance to tumor challenge or significant delay of onset of visible tumors) to some tumors of Table 1 above, especially those that were already shown to have some susceptibility to vaccination with the Vp53-wt vaccine, such as the 66.1 and CT-26 cell lines. Such tumor cell lines are expected to boost the vaccine induced immune response upon challenge.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto. The priority U. S. Patent Application No. 60/028,193 and other cited references contained herein are incorporated by reference.

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(i) APPLICANT: Wistar Institute of Anatomy & Biology
Ertl, Hildegund C.J.
Thurin, Magdalena

(ii) TITLE OF INVENTION: Methods and Compositions for Inducing a Protective Immune Respone to Cancers

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(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Howson and Howson
(B) STREET: Spring House Corporate Cntr., PO Box 457
(C) CITY: Spring House
(D) STATE: Pennsylvania
(E) COUNTRY: USA
(F) ZIP: 19477

(v) COMPUTER READABLE FORM:
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bak, Mary E.
(B) REGISTRATION NUMBER: 31,215
(C) REFERENCE/DOCKET NUMBER: WST75APCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-540-9200
(B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1317 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 136..1314

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTTGCGTTCG GGCTGGGAGC GTGCTTTCCA CGACGGTGAC ACGCTTCCCT       100

GGATTGGCAG CCAGACTGCC TTCCGGGTCA CTGCC ATG GAG GAG CCG       147
                                       Met Glu Glu Pro
                                        1

CAG TCA GAT CCT AGC GTC GAG CCC CCT CTG AGT CAG GAA ACA      189
Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr
  5                  10                  15

TTT TCA GAC CTA TGG AAA CTA CTT CCT GAA AAC AAC GTT CTG      231
Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
     20                  25                  30

TCC CCC TTG CCG TCC CAA GCA ATG GAT GAT TTG ATG CTG TCC      273
Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser
         35                  40                  45

CCG GAC GAT ATT GAA CAA TGG TTC ACT GAA GAC CCA GGT CCA      315
Pro Asp Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro
             50                  55                  60

GAT GAA GCT CCC AGA ATG CCA GAG GCT GCT CCC CCC GTG GCC      357
Asp Glu Ala Pro Arg Met Pro Glu Ala Ala Pro Pro Val Ala
                 65                  70

CCT GCA CCA GCA GCT CCT ACA CCG GCG GCC CCT GCA CCA GCC      399
Pro Ala Pro Ala Ala Pro Thr Pro Ala Ala Pro Ala Pro Ala
 75                  80                  85

CCC TCC TGG CCC CTG TCA TCT TCT GTC CCT TCC CAG AAA ACC      441
Pro Ser Trp Pro Leu Ser Ser Ser Val Pro Ser Gln Lys Thr
     90                  95                 100
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TAC CAG GGC AGC TAC GGT TTC CGT CTG GGC TTC TTG CAT TCT    483
Tyr Gln Gly Ser Tyr Gly Phe Arg Leu Gly Phe Leu His Ser
        105                 110                 115

GGG ACA GCC AAG TCT GTA ACT TGC ACG TAC TCC CCT GCC CTC    525
Gly Thr Ala Lys Ser Val Thr Cys Thr Tyr Ser Pro Ala Leu
            120                 125                 130

AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGC CCT GTG CAG    567
Asn Lys Met Phe Cys Gln Leu Ala Lys Thr Cys Pro Val Gln
                135                 140

CTG TGG GTT GAT TCC ACA CCC CCG CCC GGC ACC CGC GTC CGC    609
Leu Trp Val Asp Ser Thr Pro Pro Pro Gly Thr Arg Val Arg
145                 150                 155

GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT    651
Ala Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val
    160                 165                 170

GTG AGG CGC TGC CCC CAC CAT GAG CGC TGC TCA GAT AGC GAT    693
Val Arg Arg Cys Pro His His Glu Arg Cys Ser Asp Ser Asp
        175                 180                 185

GGT CTG GCC CCT CCT CAG CAT CTT ATC CGA GTG GAA GGA AAT    735
Gly Leu Ala Pro Pro Gln His Leu Ile Arg Val Glu Gly Asn
            190                 195                 200

TTG CGT GTG GAG TAT TTG GAT GAC AGA AAC ACT TTT CGA CAT    777
Leu Arg Val Glu Tyr Leu Asp Asp Arg Asn Thr Phe Arg His
                205                 210

AGT GTG GTG GTG CCC TAT GAG CCG CCT GAG GTT GGC TCT GAC    819
Ser Val Val Val Pro Tyr Glu Pro Pro Glu Val Gly Ser Asp
215                 220                 225

TGT ACC ACC ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TGC    861
Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn Ser Ser Cys
    230                 235                 240

ATG GGC GGC ATG AAC CGG AGA CCC ATC CTC ACC ATC ATC ACA    903
Met Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile Thr
        245                 250                 255

CTG GAA GAC TCC AGT GGT AAT CTA CTG GGA CGG AAC AGC TTT    945
Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe
            260                 265                 270

GAG GTG CGT GTT TGT GCC TGT CCT GGG AGA GAC CGG CGC ACA    987
Glu Val Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr
                275                 280
```

```
GAG GAA GAG AAT CTC CGC AAG AAA GGG GAG CCT CAC CAC GAG   1029
Glu Glu Glu Asn Leu Arg Lys Lys Gly Glu Pro His His Glu
285                 290                 295

CTG CCC CCA GGG AGC ACT AAG CGA GCA CTG CCC AAC AAC ACC   1071
Leu Pro Pro Gly Ser Thr Lys Arg Ala Leu Pro Asn Asn Thr
    300                 305                 310

AGC TCC TCT CCC CAG CCA AAG AAG AAA CCA CTG GAT GGA GAA   1113
Ser Ser Ser Pro Gln Pro Lys Lys Lys Pro Leu Asp Gly Glu
        315                 320                 325

TAT TTC ACC CTT CAG ATC CGT GGG CGT GAG CGC TTC GAG ATG   1155
Tyr Phe Thr Leu Gln Ile Arg Gly Arg Glu Arg Phe Glu Met
            330                 335                 340

TTC CGA GAG CTG AAT GAG GCC TTG GAA CTC AAG GAT GCC CAG   1197
Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys Asp Ala Gln
                345                 350

GCT GGG AAG GAG CCA GGG GGG AGC AGG GCT CAC TCC AGC CAC   1239
Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser Ser His
355                 360                 365

CTG AAG TCC AAA AAG GGT CAG TCT ACC TCC CGC CAT AAA AAA   1281
Leu Lys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys
    370                 375                 380

CTC ATG TTC AAG ACA GAA GGG CCT GAC TCA GAC TGA           1317
Leu Met Phe Lys Thr Glu Gly Pro Asp Ser Asp
        385                 390
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 393 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser
  1               5                  10                  15

Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn
                 20                  25                  30
```

Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu
35 40 45

Ser Pro Asp Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro
50 55 60

Asp Glu Ala Pro Arg Met Pro Glu Ala Ala Pro Pro Val Ala Pro
65 70 75

Ala Pro Ala Ala Pro Thr Pro Ala Ala Pro Ala Pro Ala Pro Ser
80 85 90

Trp Pro Leu Ser Ser Ser Val Pro Ser Gln Lys Thr Tyr Gln Gly
95 100 105

Ser Tyr Gly Phe Arg Leu Gly Phe Leu His Ser Gly Thr Ala Lys
110 115 120

Ser Val Thr Cys Thr Tyr Ser Pro Ala Leu Asn Lys Met Phe Cys
125 130 135

Gln Leu Ala Lys Thr Cys Pro Val Gln Leu Trp Val Asp Ser Thr
140 145 150

Pro Pro Pro Gly Thr Arg Val Arg Ala Met Ala Ile Tyr Lys Gln
155 160 165

Ser Gln His Met Thr Glu Val Val Arg Arg Cys Pro His His Glu
170 175 180

Arg Cys Ser Asp Ser Asp Gly Leu Ala Pro Pro Gln His Leu Ile
185 190 195

Arg Val Glu Gly Asn Leu Arg Val Glu Tyr Leu Asp Asp Arg Asn
200 205 210

Thr Phe Arg His Ser Val Val Val Pro Tyr Glu Pro Pro Glu Val
215 220 225

Gly Ser Asp Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn Ser
230 235 240

Ser Cys Met Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile
245 250 255

Thr Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe
260 265 270

Glu Val Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu
275 280 285

Glu Glu Asn Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro
290 295 300

Pro Gly Ser Thr Lys Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser
305 310 315

Pro Gln Pro Lys Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu
320 325 330

Gln Ile Arg Gly Arg Glu Arg Phe Glu Met Phe Arg Glu Leu Asn
335 340 345

Glu Ala Leu Glu Leu Lys Asp Ala Gln Ala Gly Lys Glu Pro Gly
350 355 360

Gly Ser Arg Ala His Ser Ser His Leu Lys Ser Lys Lys Gly Gln
365 370 375

Ser Thr Ser Arg His Lys Lys Leu Met Phe Lys Thr Glu Gly Pro
380 385 390

Asp Ser Asp

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AACGTT 6

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 390 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Thr	Ala	Met	Glu	Glu	Ser	Gln	Ser	Asp	Ile	Ser	Leu	Glu	Leu
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Pro	Leu	Ser	Gln	Glu	Thr	Phe	Ser	Gly	Leu	Trp	Lys	Leu	Leu	Pro
				20					25					30
Pro	Glu	Asp	Ile	Leu	Pro	Ser	Pro	His	Cys	Met	Asp	Asp	Leu	Leu
				35					40					45
Leu	Pro	Gln	Asp	Val	Glu	Glu	Phe	Phe	Glu	Gly	Pro	Ser	Glu	Ala
				50					55					60
Leu	Arg	Val	Ser	Gly	Ala	Pro	Ala	Ala	Gln	Asp	Pro	Val	Thr	Glu
				65					70					75
Thr	Pro	Gly	Pro	Val	Ala	Pro	Ala	Pro	Ala	Thr	Pro	Trp	Pro	Leu
				80					85					90
Ser	Ser	Phe	Val	Pro	Ser	Gln	Lys	Thr	Tyr	Gln	Gly	Asn	Tyr	Gly
				95					100					105
Phe	His	Leu	Gly	Phe	Leu	Gln	Ser	Gly	Thr	Ala	Lys	Ser	Val	Met
				110					115					120
Cys	Thr	Tyr	Ser	Pro	Pro	Leu	Asn	Lys	Leu	Phe	Cys	Gln	Leu	Val
				125					130					135
Lys	Thr	Cys	Pro	Val	Gln	Leu	Trp	Val	Ser	Ala	Thr	Pro	Pro	Ala
				140					145					150
Gly	Ser	Arg	Val	Arg	Ala	Met	Ala	Ile	Tyr	Lys	Lys	Ser	Gln	His
				155					160					165
Met	Thr	Glu	Val	Val	Arg	Arg	Cys	Pro	His	His	Glu	Arg	Cys	Ser
				170					175					180
Asp	Gly	Asp	Gly	Leu	Ala	Pro	Pro	Gln	His	Leu	Ile	Arg	Val	Glu
				185					190					195
Gly	Asn	Leu	Tyr	Pro	Glu	Tyr	Leu	Glu	Asp	Arg	Gln	Thr	Phe	Arg
				200					205					210
His	Ser	Val	Val	Val	Pro	Tyr	Glu	Pro	Pro	Glu	Ala	Gly	Ser	Glu
				215					220					225
Tyr	Thr	Thr	Ile	His	Tyr	Lys	Tyr	Met	Cys	Asn	Ser	Ser	Cys	Met
				230					235					240

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Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile Thr Leu Glu
                245                 250                 255

Asp Ser Ser Gly Asn Leu Leu Gly Arg Asp Ser Phe Glu Val Arg
                260                 265                 270

Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu Asn
                275                 280                 285

Phe Arg Lys Lys Glu Val Leu Cys Pro Glu Leu Pro Pro Gly Ser
                290                 295                 300

Ala Lys Arg Ala Leu Pro Thr Cys Thr Ser Ala Ser Pro Pro Gln
                305                 310                 315

Lys Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Lys Ile Arg
                320                 325                 330

Gly Arg Lys Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu
                335                 340                 345

Glu Leu Lys Asp Ala His Ala Thr Glu Glu Ser Gly Asp Ser Arg
                350                 355                 360

Ala His Ser Ser Tyr Leu Lys Thr Lys Lys Gly Gln Ser Thr Ser
                365                 370                 375

Arg His Lys Lys Thr Met Val Lys Lys Val Gly Pro Asp Ser Asp
                380                 385                 390
```

WHAT IS CLAIMED IS:

1. A pharmaceutical or vaccine composition comprising:

a recombinant vector comprising a mutant p53 gene encoding a biologically inactive p53 product, said gene under the regulatory control of a promoter which directs the expression of said gene product in a mammalian subject to produce an immunologic response, and

a suitable pharmaceutical carrier.

2. The composition according to claim 1, wherein the vector is an expression vector.

3. The composition according to claim 1, wherein the vector is a recombinant virus.

4. The composition according to any of claims 1 to 3, wherein said mutant p53 gene carries a mutation in the tetramerization domain which causes functional inactivation of said p53.

5. The composition according to claim 4, wherein said gene is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

6. The composition according to any of claims 1 to 6, further comprising a chemokine, cytokine, or combinations thereof which enhances the immune response to said p53 gene product following administration of the composition to a mammal.

7. A pharmaceutical or vaccine composition comprising:

a DNA molecule which induces an immune response to a tumor cell comprising a DNA sequence encoding a biologically inactive p53 gene product under the regulatory control of a promoter which directs the expression of said gene product in a mammalian subject, and

a suitable pharmaceutical carrier.

8. The composition according to claim 7, wherein said DNA sequence is a mutant p53 gene having an altered p53 tetramerization domain which causes functional inactivation of said p53.

9. The composition according to claim 7 or claim 8, wherein said gene is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

10. The composition according to any of claims 7 to 9 further comprising a chemokine, cytokine or combinations thereof which enhances the immune response to said p53 gene product following administration of the composition to a mammal.

11. The composition according to claim 10, wherein said chemokine, cytokine or combinations thereof is in the form of a plasmid vector carrying a DNA sequence encoding said chemokine, cytokine or combinations thereof under the regulatory control of a promoter which directs the expression of said chemokine, cytokine or combinations thereof in a mammalian subject.

12. A recombinant vector comprising a p53 gene fused to a signal sequence under the regulatory control of a promoter which directs the expression of said fusion sequence in a mammalian subject, said signal sequence retargeting said p53 gene product toward the endosomal or lysosomal pathway of the cell.

13. The vector according to claim 12, which is an expression vector.

14. The vector according to claim 12, which is a recombinant virus.

15. The vector according to any of claims 12 to 14, wherein said p53 gene carries a mutation in the tetramerization domain which causes functional inactivation of said p53.

16. The vector according to any of claims 12 to 14, wherein said p53 gene is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

17. A pharmaceutical or vaccine composition comprising a vector according to any one of claims 12-16 in a suitable pharmaceutical carrier.

18. The composition according to claim 17 further comprising a chemokine, cytokine or combinations thereof which enhances the immune response to said p53 gene product following administration of the composition to a mammal.

19. A method for inducing an immune response to a tumor cell in a mammal comprising the step of co-administering to said mammal:

(a) a recombinant virus vector comprising a p53 gene under the regulatory control of a promoter which directs the expression of said gene product, said

virus capable of becoming toxic to an infected cell prior to apoptosis of said cell caused by said gene product; and

(b) an optional suitable amount of a chemokine, cytokine or combinations thereof that enhances an immune response.

20. The method according to claim 19, wherein said p53 gene is mutated or modified.

21. The method according to claim 20, wherein said p53 gene encodes a biologically inactive form of p53.

22. A method for inducing an immune response to a tumor cell in a mammal comprising the step of co-administering to said mammal:

(a) a recombinant vector comprising a mutant p53 gene encoding a biologically inactive p53 product under the regulatory control of a promoter which directs the expression of said gene product, said virus capable of persistently infecting a cell; and

(b) an optional suitable amount of a chemokine, cytokine or combinations thereof.

23. A method of inducing an immune response to a tumor cell in a mammal comprising the step of administering to a subject a pharmaceutical or vaccine composition comprising:

a DNA molecule which induces an immune response to a tumor cell comprising a DNA sequence encoding a biologically inactive p53 gene product under the regulatory control of a promoter which directs the expression of said gene product in a mammalian subject.

24. The method according to claim 23, further comprising the step of administering to said mammal a chemokine, cytokine or combinations thereof.

25. The method according to claim 24, wherein said chemokine, cytokine or combinations thereof is administered in plasmid vector carrying a DNA sequence encoding said chemokine, cytokine or combinations thereof under the regulatory control of a promoter which directs expression of said chemokine, cytokine or combinations thereof in a mammalian cell.

26. The method according to any of claims 23 to 25, wherein said chemokine, cytokine or combinations thereof is administered to said subject after the administration of said DNA encoding said p53 antigen.

27. The method according to any of claims 23 to 25, wherein said chemokine, cytokine or combinations thereof is administered to said subject simultaneously with the administration of said DNA encoding said p53 antigen.

28. The method according to any of claims 23 to 27, where said gene is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

29. A method for inducing an immune response to a tumor cell comprising the steps of administering to said mammal a recombinant vector of claims 12 to 16; and an optional amount of a chemokine, cytokine or combinations thereof.

30. A method for inducing an immune response to a tumor cell comprising the steps of:

(a) first administering to a subject a suitable amount of a DNA sequence encoding a p53 antigen; and

(b) subsequently boosting said subject with a suitable amount of a recombinant vector, said vector comprising a p53 gene under the regulatory control of a promoter which directs the expression of said gene product.

31. The method according to claim 30 further comprising co-administering a chemokine, cytokine or combinations thereof.

32. The method according to claim 31, wherein said chemokine, cytokine or combinations thereof is administered as DNA, as protein or in a plasmid vector.

33. The method according to claim 31 or claim 32, wherein said chemokine, cytokine or combinations thereof is administered simultaneously with, or after said first or booster administration.

34. The method according to any of claims 30 to 33, wherein said p53 gene of step (a) or (b) is a mutant p53 gene encoding a biologically inactive form of p53.

35. The method according to any of claims 30 to 33, wherein said p53 gene of step (a) or (b) is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

36. The method according to any of claims 30 to 34, wherein said gene of step (a) or (b) is a p53 gene is fused to a signal sequence under the regulatory control of a promoter which directs the expression of said fusion sequence in a mammalian subject, said signal sequence retargeting said p53 gene product toward the endosomal or lysosomal pathway of the cell.

37. Use of a recombinant virus vector comprising a p53 gene under the regulatory control of a promoter which directs the expression of said gene product, said virus capable of becoming toxic to an infected cell prior to apoptosis of said cell caused by said gene product in the preparation of a medicament for inducing an immune response to a tumor cell in a mammal, wherein said medicament is optionally administered to said mammal with a suitable amount of a chemokine, cytokine or combinations thereof that enhances an immune response.

38. Use according to claim 37, wherein said p53 gene is mutated or modified.

39. Use according to claim 37, wherein said p53 gene encodes a biologically inactive form of p53.

40. Use of a recombinant virus vector comprising a mutant p53 gene encoding a biologically inactive p53 product under the regulatory control of a promoter which directs the expression of said gene product, said virus capable of chronically infecting a cell without becoming toxic thereto in the preparation of a medicament for inducing an immune response to a tumor cell in a mammal, wherein the medicament is optionally co-administered to said mammal with a suitable amount of a chemokine, cytokine or combinations thereof.

41. Use of a DNA molecule comprising a DNA sequence encoding a biologically inactive p53 gene product under the regulatory control of a promoter which directs the expression of said gene product in mammalian cells in the preparation of a medicament which induces an immune response to a tumor cell.

42. Use according to claim 41, wherein said medicament is co-administered with a chemokine, cytokine or combinations thereof.

43. Use according to claim 42, wherein said chemokine, cytokine or combinations thereof is administered in plasmid vector carrying a DNA sequence encoding said chemokine, cytokine or combinations thereof under the regulatory control of a promoter which directs expression of said chemokine, cytokine or combinations thereof in a mammalian cell.

44. Use according to claim 42 or claim 43, wherein said chemokine, cytokine or combinations thereof is administered to said subject after the administration of said DNA encoding said p53 antigen.

45. Use according to claim 42 or claim 43, wherein said chemokine, cytokine or combinations thereof is administered to said subject simultaneously with the administration of said DNA encoding said p53 antigen.

46. Use according to any of claims 41 to 845 where said gene is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

47. Use of a recombinant vector comprising a p53 gene under the regulatory control of polynucleotide sequences which direct the expression of said gene product in the preparation of a medicament for boosting an immune response to a tumor cell, wherein said medicament is administered following administration of an immunogenic composition comprising a DNA sequence encoding a p53 antigen.

48. Use according to claim 84, 47erein said medicament is further administered with a chemokine, cytokine or combinations thereof.

49. Use according to claim 48, wherein said chemokine, cytokine or combinations thereof is administered as DNA, as protein or in a plasmid vector.

50. Use according to claim 48 or claim 49, wherein said chemokine, cytokine or combinations thereof is administered simultaneously with, or after, said medicament.

51. Use according to any of claims 47 to 50, wherein said p53 gene is a mutant p53 gene encoding a biologically inactive form of p53.

52. Use according to any of claims 47 to 50, wherein said p53 gene is a mammalian p53 having a mutation at the codon encoding the amino acid residue homologous to residue 338 of murine p53.

53. Use according to any of claims 47 to 50, wherein said vector is capable of delivering said gene to said subject without becoming toxic thereto and said p53 gene encodes a biologically inactive form of p53.

54. Use according to any of claims 47 to 52, wherein said vector is a virus capable of becoming toxic to an infected cell prior to apoptosis of said cell caused by said gene product and said p53 encodes a wild-type p53.

55. Use according to any of claims 46 to 53, wherein said p53 gene is fused to a signal sequence under the regulatory control of a promoter which directs the expression of said fusion sequence in a mammalian subject, said signal sequence retargeting said p53 gene product toward the endosomal pathway of the cell.

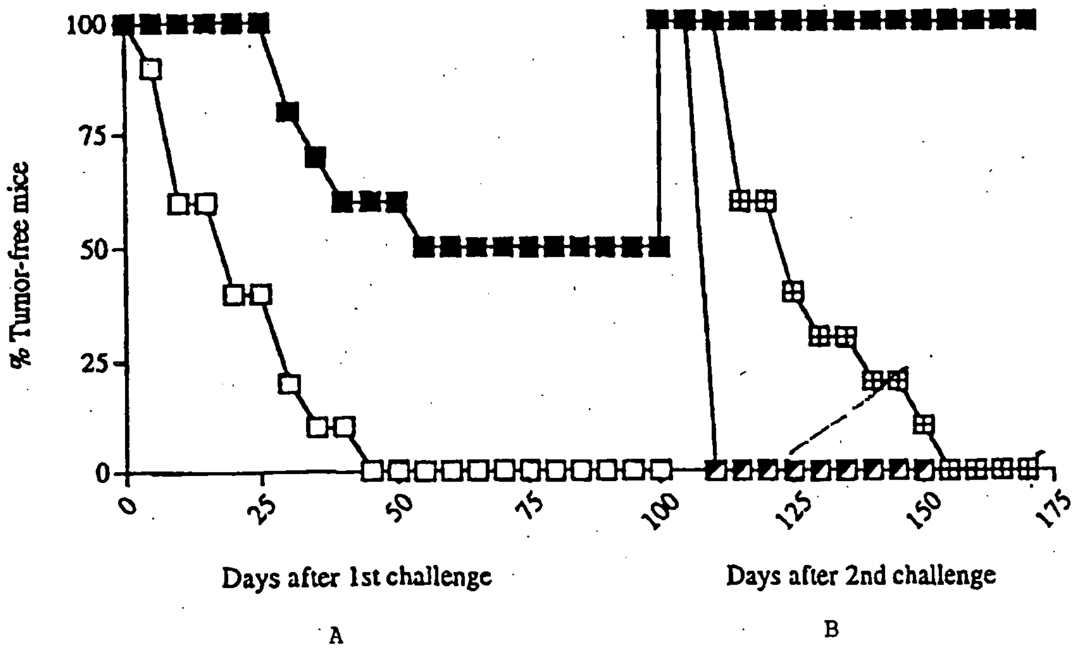
100
75
50
25
0
% Tumor-free mice
0
25
50
75
100
125
150
175
Days after 1st challenge
Days after 2nd challenge
A
B

FIG. 1

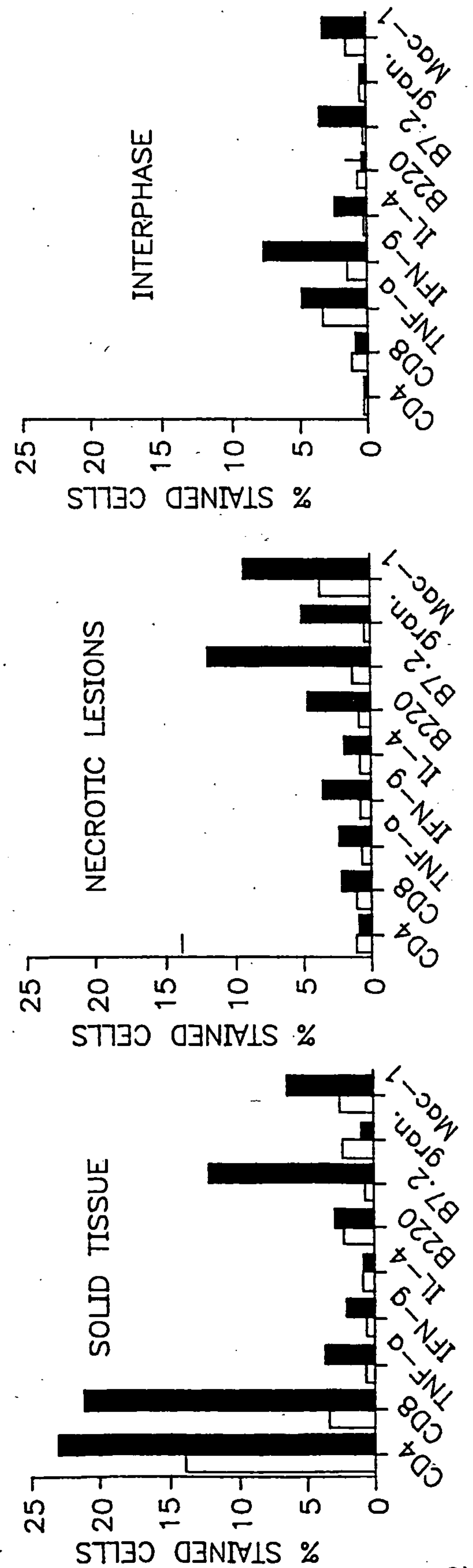

FIG. 2C

FIG. 2B

FIG. 2A

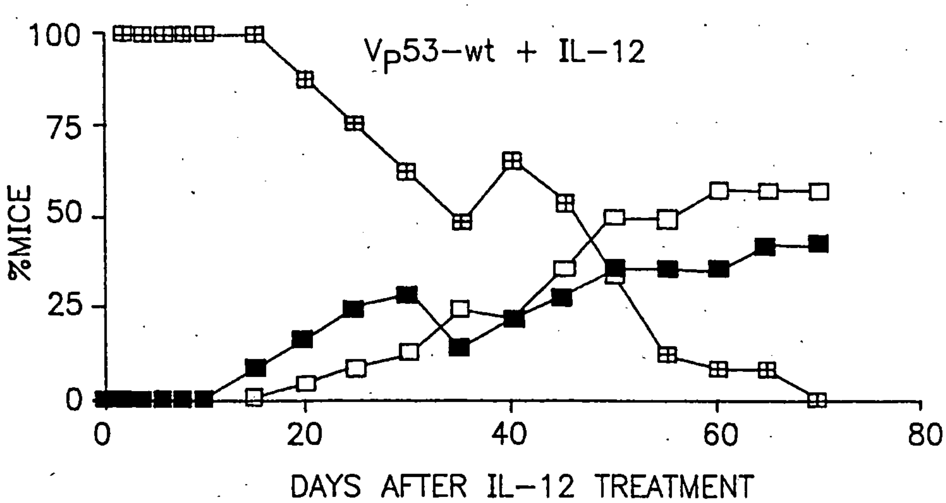
100
75
50
25
0
%MICE
V_P53-wt + IL-12
0
20
40
60
80
DAYS AFTER IL-12 TREATMENT

FIG. 3